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CORRECTIONS.

On page 125, Vol. LIV, No. 1, September, 1922, the following foot-note should be inserted at the bottom of the page.

"This graph was intended only to show errors in measurements and not absolute values. If used to read pH from observed CO₂ content of blood or plasma and CO₂ tension and if one accepts pK' for whole blood = 6.15 and pK' for serum = 6.10, then a correction for whole blood of +0.04 pH must be added and for plasma or serum of -0.04 pH. The diagram would otherwise appear to indicate that the pH of whole blood is 0.08 lower than its true plasma at the same CO₂ tension. The pH of whole blood, however, is by definition the pH of its true plasma at the same CO₂ tension."

On page 593, Vol. LVI, No. 2, June, 1923, the asterisk (*) foot-note should read "*Aided by a grant from the Edward N. Gibbs Fund*" instead of "*Aided by a grant from the Robert M. Girvin Foundation.*"

On page 256, Vol. LVII, No. 1, August, 1923, line 16, for "*later*" read "*also.*"

On page 257, line 6, for "*placed together in*" read "*both were treated with.*"

On page 284, 3rd line from the bottom of the page, for "*H. Wooghoudt*" read "*H. Hooghoudt.*"

2nd line from the bottom of the page, for "*W. Gieteling*" read "*H. Gieteling.*"

In the Journal for November, 1923, in the paper by Underhill and Gross the decimal point in each tabulated figure for Na should be moved one point to the right.

On page 791, Vol. LVIII, No. 3, January, 1924, the signs of the last two terms of equation (15) should be reversed. The corrected equation is

$$[\text{CO}_2]_p = [\text{CO}_2]_b + \left\{ (0.0159[\text{CO}_2]_b - 0.281)h - \left(\frac{P_{\text{CO}_2}}{40} \times 2.85 \right) + 2.85 \right\}$$

ALKALOSIS, SODIUM POISONING, AND TETANY.

By ISIDOR GREENWALD.

(From the Harriman Research Laboratory, Roosevelt Hospital, New York.)

(Received for publication, November 26, 1923.)

In a paper entitled "The supposed relation between alkalosis and tetany," the author (1) reported the results of a series of experiments upon dogs receiving intravenous injections of sodium carbonate or bicarbonate. It was found that it required large amounts of these substances to produce convulsions and that the concentration of sodium in the plasma at that time was approximately the same as in other dogs in whom convulsions were induced by the injection of sodium chloride or sulfate. A specific connection between an increased alkalinity of the blood and the appearance of convulsions was denied.

In a recent paper, Denis and von Meysenbug (2) have reported the results of a repetition of these experiments upon dogs anesthetized with ether and have confirmed the author's statement as to the concentration of sodium in the plasma of dogs in convulsions after the administration of sodium bicarbonate, chloride, or sulfate. However, they differentiate between two kinds of convulsions, those following injections of sodium bicarbonate being due to "tetany," whereas those following injections of either sodium chloride or sulfate are not. The reason for this distinction lies in the differences they observed in the responses to electrical stimulation. The dogs receiving sodium bicarbonate are said to have become hyperexcitable, the others not.

Denis and von Meysenbug¹ state:

" . . . it is, we believe, generally conceded by both clinicians and physiologists that it is practically impossible to state with absolute certainty whether convulsions observed in man or in animals are or are not due to tetany without a determination of the electrical reactions."

¹ Denis and von Meysenbug (2), p. 48.

It is true that clinicians do generally diagnose as tetany only those convulsive or spastic conditions in which an increased electrical excitability can be demonstrated. But this is really nothing more or less than a matter of definition. Cases showing electrical hyperexcitability are those of tetany; those that do not, are not so classified. "Tetany" is not a clinical entity. There is as yet no satisfactory evidence that tetania strumipriva (or parathyreopriva), idiopathic tetany, and gastric tetany, to mention only three types, have any relation whatever to one another, except for the resemblance between certain of the symptoms. As a matter of fact, clinicians do recognize cases of tetany without electrical hyperexcitability. Thus Falta writes:²

"Kahn and I observed a case of chronic tetany with acute exacerbations, in which all the important symptoms of tetany were present pronouncedly, but Erb's phenomenon, in spite of the presence of severe spasms, was absent during the first days."

Holmes³ states:

"1. The appearance of cathodal opening contractions under 5 ma. (and in the absence of certain conditions already mentioned) in children under 5 years of age is pathognomonic of tetany. Cathodal opening contractions are, however, not infrequently absent in cases of clinical tetany.

"2. The appearance of anodal opening contractions with less current than that causing anodal closing contractions, and under 5 ma. during the first 6 months of life is probably pathognomonic of tetany in all cases; their appearance with less current than that causing anodal closing contractions and under 2 ma. is probably pathognomonic up to the fourth or fifth year; thereafter it is of little significance."

There seems to be no reason whatever for the inclusion of the words "or in animals" in the statement of Denis and von Meysenbug. Most of those working on animals, as indeed many of those working with men, have been apt to use the word "tetany" to denote a spastic condition of the muscles, which is accompanied or followed by tremor, twitching, and tonic-clonic convulsions. And this use of the word seems to have been countenanced by at least some of those clinicians who have had occasion to refer to the animal experiments. Thus Howland and Marriott (5), refer-

²Falta (3), p. 178.

³Holmes (4), p. 29.

ring to the experiments of Binger (6) state that these "show that typical tetany may be produced in dogs when the parathyroids are left entirely undisturbed." Binger did not test the electrical reactions of his animals.

Denis and von Meysenbug neglect to mention the fact that MacCallum and his coworkers (7) had previously tested the electrical reactions of dogs receiving injections of sodium carbonate. They had found that, although the electrical excitability was sometimes increased, this was not always the case, although the other symptoms, such as tremors and convulsions, did not differ from those observed when the electrical excitability was increased.

Just what is to be considered electrical hyperexcitability? Denis and von Meysenbug⁴ state:

"Reactions which are considered characteristic of tetany or spasmodophilia are those showing either anodal reversal ($AOC < ACC$ and < 5 milliamperes) or $COC < 5$ milliamperes or $CCTe = 5$ milliamperes."

The statement of Holmes, which is at least partially contradictory, has already been quoted. But, after all, we are dealing here, not with children, whether 6 months, 2 years, or 5 years old or older, but with dogs; and comparisons must be made with dogs. As far as the author is aware the only previous workers who have tested the electrical excitability of dogs are MacCallum and Paton and their respective associates.

The former (8) wrote:

"Bekanntlich bildet die erhöhte elektrische Erregbarkeit der motorischen Nerven das am leichtesten zu erkennende und konstanteste Symptom der Tetanie. Die Leichtigkeit, mit der eine Reaktion auf die Kathodenöffnung hervorgerufen werden kann, ist besonders charakteristisch, während die Veränderungen in der Erregbarkeit bei Kathoden- und Anodenschliessung und selbst die Anodenöffnungszuckung, obgleich sie gewöhnlich der Kathodenöffnungszuckung parallel geht, weder diagnostisch so wichtig noch so umfangreich sind.

"Beim Hunde tritt in gesunden Zustände gewöhnlich keine Kathodenöffnungszuckung bei Strömen unter 5 Milliampères auf, während die Anodenöffnungszuckung bei einem Strom von 1, 5–4 Ma. und die Kathoden- und Anodenschliessungszuckung bei Strömen unter 1 Ma. auftreten können."

⁴Denis and von Meysenbug (2), p. 49.

MacCallum seems not to have anesthetized his dogs. Certainly his figures for the strength of current normally required to elicit contractions are much lower than those of Denis and von Meysenbug.

Paton and his associates anesthetized their dogs, though only for a short time, before testing the electrical reactions. Their values for the normal excitability are of the same order as those of Denis and von Meysenbug. In view of the importance that Denis and von Meysenbug attach to "anode reversal," it is interesting to read in the paper by Paton, Findlay, and Watson:⁵

"The normal response of nerve and muscle varies greatly. The usual sequence is K.C.C., A.C.C., A.O.C., and K.O.C., but not infrequently A.O.C. is more marked than A.C.C."

And again,⁶ under the heading "Tetania parathyreopriva and idiopathic tetany:"

"As a rule no alteration of the normal sequence of the various responses results, but the A.O.C. is more frequently than in health greater [lower in sense of Denis and von Meysenbug—I.G.] than A.C.C., and on occasions may equal or be greater than K.C.C. This is, however, of no diagnostic value."

Since the animals used by Denis and von Meysenbug were anesthetized with ether for periods of from $2\frac{1}{2}$ to $6\frac{1}{2}$ hours, the following quotation from Paton, Findlay, and Watson is not without significance:⁶

"Ether may cause a transient increase which may be followed after about 15 minutes by a slow progressive decrease."

Just as did MacCallum, Paton and his associates regarded the lowering of the strength of current required to elicit the cathodal opening contraction as the most accurate indication of tetany. *Of the five experiments with sodium bicarbonate, reported by Denis and von Meysenbug, the cathodal opening contraction was practically, if not entirely, unaffected in four and, in the fifth, the determination was made impossible because of cathodal closing tetanus at 4.5 or 6 milliamperes.*

⁵Paton, Findlay, and Watson (9), p. 312.

⁶Paton, Findlay, and Watson (9), p. 313.

In view of all these inconsistencies and contradictions, it seems to the author that the work of Denis and von Meysenbug can only be regarded as a confirmation of his conclusion that the convulsions after injections of sodium carbonate or bicarbonate are not due to alkalosis but to "sodium poisoning," a disturbance due to excess of sodium ion of the normal relations between this and other cations. They also indicate that the symptoms observed after the injection of alkalies have nothing to do with tetania parathyreopriva and give additional support to the view previously expressed by the author:*

"Tetany and convulsions are not due to any single cause. Any one of a multitude of disturbances in the equilibrium within certain tissues may be responsible. Convulsions are to be regarded as a sign of approaching or partial disintegration of the neuromuscular apparatus. The defect may occur in any one of several structures and may be due to any one of many causes."

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*Greenwald (1), p. 298.

THE RESOLUTION OF INACTIVE MALIC ACID INTO OPTICALLY ACTIVE FORMS.

By H. D. DAKIN.

(*Scarborough-on-Hudson.*)

(Received for publication, December 13, 1923.)

It is a curious fact that, hitherto, inactive malic acid has never been satisfactorily resolved into its active components. While the levo acid is of course accessible from natural sources, the dextro acid has been difficult to prepare. In a recent paper McKenzie and Plenderleith (1) describe the indirect preparation of *d*-malic acid by resolving $\gamma\gamma\gamma$ -trichloro- β -hydroxybutyric acid by means of quinine and then acting on the levo acid with alkali. This method was the only one known which was reasonably convenient. The earlier indirect methods for obtaining *d*-malic acid are as follows:

(a). The reduction of *d*-tartaric acid by hydriodic acid (Bremer, 2). Freudenberg (3) has shown this method to be useless for preparative work.

(b). The action of nitrous acid on *d*-asparagine (Piutti, 4).

(c). The oxidation of *d*- α -hydroxybutyrolactone with nitric acid (Nef, 5; Glattfeld and Miller, 6).

(d). The action of silver oxide and water on *d*-chloro- and *d*-bromosuccinic acids (Walden, 7). Fumaric acid and inactive malic acid are formed simultaneously and the pure dextro acid could not be isolated. Similar results were obtained by Holmberg (8) who used both *d*- and *l*-halogen derivatives of succinic acid.

(e). *d*-Tartaric acid dimethyl ester on monacetylation gives with thionyl chloride and pyridine, an acetyl derivative of chloromalic acid, which on hydrolysis and reduction yields *d*-malic acid (Freudenberg and Brauns, 9).

The only direct method recorded for the preparation of *d*-malic acid is that due to Bremer (10) who crystallized the acid cinchonine salt of inactive malic acid and obtained a small quantity of dextro-rotatory ammonium hydrogen malate. Pictet (11) was completely unable to resolve malic acid by this method

and McKenzie and Plenderleith describe the method as unsuitable so far as their experiments go. Since the difference in solubility in water of the cinchonine *d*- and *l*-malates is very slight the method as outlined by Bremer is certainly not a practical one.

In some recent experiments bearing on the formation of malic acid by fermenting yeast, the writer's attention was drawn to cinchonine salts for the identification of malic acid, following the suggestion of Lindet (12). A few preliminary experiments with active and inactive malic acids quickly showed that the method while useful for the identification of *l*-malic acid was useless for the dextro acid. Further experiments indicated an easy direct resolution of inactive malic acid.

The method is based on the following facts: The acid cinchonine salt of *l*-malic acid is sparingly soluble in cold methyl alcohol (2 per cent) and even less soluble in acetone (0.5 per cent), while the salt of the dextro acid is extremely soluble in both methyl alcohol and acetone. Starting with inactive malic acid, at least 90 per cent of the levo acid may be separated in a single operation by crystallizing the cinchonine salt from methyl alcohol or acetone. The filtrate on evaporating off the organic solvent and replacing it by water gives an almost equal yield of pure cinchonine *d*-malate which crystallizes readily and has a solubility in water of about 1.7 gm. per 100 cc. at 10°. Both salts are almost optically pure as first obtained, but may be readily recrystallized with but slight loss.

Since inactive malic acid is now available as a very cheap commercial product, the present method is undoubtedly the most convenient for the preparation of the active malic acids. While presenting nothing new in principle, the method of resolution is unusual since the same alkaloid is deliberately chosen for the separation of both active components, but using different solvents in the two cases. It is usually necessary to resort to different alkaloids in order to secure well crystallized salts of each of the active forms. The whole process of resolution is so exceptionally easy to carry out and the materials are so readily accessible that it might well serve as a student's exercise.

EXPERIMENTAL.

Cinchonine l-Malate.—Cinchonine (29.4 gm.) and inactive malic acid (13.4 gm.) in molecular proportions are dissolved by heating to boiling with 130 cc. of technical methyl alcohol. The solution was allowed to cool slowly and eventually placed in a cold place overnight. Crystallization takes place very readily in the form of opaque, rather soft masses of crystals which, under the microscope, appear as stout prisms. They are filtered off and washed with a little cold methyl alcohol. The yield of cinchonine *l*-malate varies from 19.0 to 19.7 gm., compared with a theoretical yield of 21.4. As already stated, acetone may be used equally as well as methyl alcohol as solvent. It is well to dissolve the malic acid first in boiling acetone and then add the powdered cinchonine. The *l*-malate separates at once.

The crude product is surprisingly free from impurities and on decomposition it gave *l*-malic acid, which on treatment with uranium acetate showed a specific rotation of -470° compared with -482° for the perfectly pure acid, under similar conditions. The dextro-rotation ($+574^\circ$) observed after addition of ammonium molybdate was equal to that recorded by McKenzie and Plenderleith for the pure levo acid ($+568^\circ$), using the conditions prescribed by these authors.

The complete purification of cinchonine *l*-malate may be effected either by crystallization from about 10 parts of boiling methyl alcohol or, even better, by crystallization from boiling water. The salt separates from the latter solvent in clear hard prisms. The salt, crystallized from either solvent, retains no water of crystallization and melts sharply at $197-198^\circ$ (uncorrected). It dissolves readily in hot water, but requires about 50 parts of cold water (10°) for solution. Unlike the salt of the dextro acid, it is very sparingly soluble in acetone even when boiling. At 7° its solubility in cold acetone is about 0.465 per cent. It is practically insoluble in chloroform and benzene. Its specific rotation was observed in aqueous solution:

$$c = 2.0; \quad l = 2.2; \quad \alpha = +6.37^\circ$$

$$[\alpha]_D^{19} = +146^\circ$$

Cinchonine d-Malate.—The methyl alcoholic or acetone filtrate from the levo salt is concentrated on the water bath, adding water occasionally to replace the alcohol. After all the methyl alcohol or acetone has been removed the residue is dissolved in about 90 cc. of hot water and allowed to crystallize in a cool place. Crystallization of the *d*-malate takes place readily in the form of fine rosettes of clear silky prismatic needles. The salt, after filtering and washing with a little cold water, is practically pure and, after drying in warm air, weighs 22 gm. It retains 2 molecules of water of crystallization so that the yield of anhydrous salt is 20.2 gm.

In order to test the purity of the salt, some of it was converted into malic acid without further purification. On treatment with uranium acetate the acid showed $[\alpha]_D^{18} = -488^\circ$, indicating complete optical purity.

Cinchonine *d*-malate may be best recrystallized from hot water in which it is freely soluble. It is sparingly soluble in cold water, a saturated solution at 10° retaining about 1.7 per cent of the salt.

Its water of crystallization is retained with some tenacity until a temperature of about 95° is reached when it is completely lost.

0.9462 gm. lost 0.0742 gm. H_2O *in vacuo* at $95-97^\circ = 7.84$ per cent H_2O .

$C_{15}H_{22}N_2O \cdot C_4H_5O_6 \cdot 2H_2O$ requires 7.76 per cent H_2O .

It is extremely soluble in methyl alcohol and acetone, but sparingly soluble in chloroform and insoluble in benzene. The anhydrous salt may be crystallized from a mixture of acetone and chloroform, but water is a far better solvent for the purpose.

Cinchonine *d*-malate, unlike the levo salt, possesses no definite melting point. The salt, containing water of crystallization, begins to melt indefinitely around 106° while the anhydrous salt melts indefinitely to a turbid wax-like mass at $125-135^\circ$, but does not become perfectly clear until about 150° .

The rotation of the salt was observed in aqueous solution:

$$c = 2.31 \text{ (anhydrous salt)}; \quad l = 2.0; \quad \alpha = +7.06^\circ$$

$$[\alpha]_D^{18} = +153^\circ$$

d- and l-Malic Acids.—In order to recover the active malic acids from their cinchonine salts they were dissolved in hot water and the alkaloid was removed by precipitation with ammonia. In the earlier experiments the filtrate was precipitated with basic lead acetate and the lead malate in aqueous suspension decomposed with hydrogen sulfide. This method is by no means quantitative and it was eventually replaced by ether extraction. The ammonium malate solution was concentrated to a thin syrup, acidified with phosphoric acid, and then extracted with ether in an apparatus for rapid continuous extraction. The whole of the acid is readily recovered by 24 hours extraction. The ether residue is best dissolved in a little hot water and concentrated in a desiccator. Crystallization is readily effected. Both forms melted at 99–100°.

The optical rotation in water was observed in 7 per cent solution. The values obtained, $[\alpha]_D^{18} = +2.33^\circ$ and $[\alpha]_D^{18} = -2.31^\circ$, agree closely with previous observations.

The enhanced rotation induced by uranium salts was observed under the following conditions. The active acid (about 0.1 gm.) was exactly neutralized with sodium hydroxide, and then 1 drop of acetic acid together with 0.5 gm. of powdered uranium acetate. The whole was diluted to 20 cc. and allowed to stand 1 hour.

Dextro Acid. $c = 0.5$; $l = 2.2$; $\alpha = +5.32^\circ$

$$[\alpha]_D^{18} = +483^\circ$$

Levo Acid. $c = 0.5673$; $l = 2.2$; $\alpha = -6.02^\circ$

$$[\alpha]_D^{18} = -482^\circ$$

l-Malic acid derived from natural sources gave identical values. The effect of ammonium molybdate on the active acids was also observed. Darmois (13) has examined the crystalline ammonium dimolybdo-*l*-malate and later in conjunction with Périn (14) the corresponding *d*-malate, $C_4H_6O_5 \cdot 2MoO_3 \cdot 2NH_3$. These compounds have a high optical rotation in the opposite sense to that of the contained malic acid. McKenzie and Plenderleith have made use of this fact for the examination of their active malic acids. They treated 0.2233 gm. of the levo acid with

0.5152 gm. of "ammonium molybdate" and observed a specific rotation calculated for the malic acid of $+568^\circ$ at 15.5° . My own observation using similar quantities gave $[\alpha]_D^{18}$ of from $+564^\circ$ to $+570^\circ$ for the levo acid and -566° to -572° for the dextro acid. But it was noted that the proportion of ordinary ammonium molybdate, $\text{Mo}_7\text{O}_{24}(\text{NH}_4)_6 \cdot 4\text{H}_2\text{O}$, apparently used by McKenzie and Plenderleith was insufficient for the complete formation of Darmois' compound. On increasing the proportion of the molybdate up to double that quoted specific rotations of from 696° to 705° were observed. On increasing the molybdate concentration fivefold the figures fell to about 649° .

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NUTRITION AND GROWTH ON DIETS HIGHLY DEFICIENT OR ENTIRELY LACKING IN PREFORMED CARBOHYDRATES.*

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The use of insulin in controlling diabetes has made any additional information respecting the carbohydrates in nutrition a subject of immediate interest. The normal content of glucose in the blood is ordinarily very constant. When for any reason this percentage of sugar is decreased symptoms of acute distress promptly arise. For example, after unduly large doses of insulin marked hypoglycemia results, promptly manifesting itself by signs of exhaustion. Similar symptoms of depression and collapse attend the hypoglycemia induced by extirpation of the liver. Administration of glucose speedily brings relief in both cases.

Furthermore, it now appears that the ability of the organism to metabolize fats is in some way dependent upon an adequate supply of physiologically available carbohydrate. Without the latter, ketosis may ensue. According to the current conceptions more or less definite ratios must exist between the ketogenic and the antiketogenic factors in the metabolism, if ketosis or the appearance of "acetone substances" is to be averted. Sugar is preeminent as the antiketogenic or ketolytic substance in nutrition; and the effect of carbohydrate in preventing or abolishing ketonemia is believed to be the result of definite chemical reactions in the tissues.

* The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, D. C.

It is today readily conceivable that sugar can become available in metabolism without having been supplied as *preformed* carbohydrate in the diet. During starvation the sugar content of the blood is maintained at the normal level despite the lack of an exogenous supply. Under such conditions the blood sugar may arise from depots of glycogen in the body; but there are experimental proofs that sugar can arise endogenously from amino acids in protein breakdown as well as through glycogenolysis. The glycerol radical of the fats is a further potential source of glucose, or at least of antiketogenic substance.

Whether these purely endogenous precursors—tissue glycogen, amino acids, glycerol—will suffice in the long run to permit a normal metabolism has not been demonstrated. Their adequacy might well depend upon the extent to which fats and proteins respectively dominate as sources of energy when good carbohydrate is not available. Thus the physiological consequences of diets relatively rich in fats and poor in protein or *vice versa* might be different, it being conceivable that an abundance of fat might entail a lack of the antiketogenic factor requisite for its proper utilization. This seems to be true in certain cases of diabetes.

The problems at issue are open to experimental test by the methods of animal feeding which have been developed in recent years. We have already reported very briefly some of our earliest observations under conditions designed to ascertain whether the nutrition of growth requires preformed carbohydrate in the diet.¹ These indicate a surprising capacity in rats to grow to adult size at a normal rate on rations in which the amount of digestible carbohydrate was at most exceedingly small.

In the present paper we give the result of feeding experiments that offer further evidence of the relation of the composition of the food to the production of sugar in the body.

Experimental Procedures.—Young albino rats were fed on food mixtures including more or less purified protein materials—casein, edestin, lactalbumin, “meat residue,” etc.—a mixture of inorganic salts long in use in our feeding trials,² and some

¹ Osborne, T. B., and Mendel, L. B., *Proc. Soc. Exp. Biol. and Med.*, 1920–21, xviii, 136; *Proc. Nat. Acad. Sc.*, 1921, vii, 157.

² Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxvii, 572.

source of vitamins, usually dried alfalfa,³ butter fat, or cod liver oil for A and dried brewery yeast⁴ or the Osborne-Wakeman "yeast fraction" II⁵ for B. The growth of the animals was used as an index of their nutritive condition. We shall discuss here only experiments in which the animals by growth at least trebled or quadrupled the weight that they exhibited at the beginning of the feeding trials. It seems unlikely that rats can grow vigorously to 250 gm. and upward in body weight while their metabolism is distinctly abnormal. Hence we accept the criterion of growth as evidence of satisfactory nutrition for our present purposes. In the graphs reproduced in this paper the changes in body weight to the maximum size attained in each test are shown. The methods of feeding followed the routine of our laboratories as outlined by Ferry.⁶

*Experiments with Diets Extremely Poor in Both Carbohydrates
and Fats and Consequently Very Rich
in Proteins.*

Young rats were placed upon a diet consisting of meat residue⁷ 90 per cent, gliadin 5 per cent, salts 5 per cent, together with daily additions of yeast, 200 mg., and alfalfa, 400 mg. The gliadin was included because its adhesive properties helped to bind together the various ingredients of the diet. In Period 2 meat residue alone (95 per cent) was the source of the protein.

The air-dry "meat residue" contained about 14 per cent N = 87.5 per cent protein ($N \times 6.25$). The carbohydrate (glycogen) content must have been negligible because the preparation had been extracted thoroughly with hot water. Consequently the only sources of appreciable preformed carbohydrate were the dried yeast and alfalfa used as vitamin-bearing supplements and "roughage." An estimation of the maximum amount of digestible carbohydrate furnished by the small quantities of these supplements indicates that they would not exceed 1 gm.

³ Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1920, xli, 549.

⁴ Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1922, liv, 739.

⁵ Osborne, T. B., and Wakeman, A. J., *J. Biol. Chem.*, 1919, xl, 383.

⁶ Ferry, E. L., *J. Lab. and Clin. Med.*, 1920, v, 735.

⁷ For the preparation of this product see Osborne, T. B., Wakeman, A. J., and Ferry, E. L., *J. Biol. Chem.*, 1919, xxxix, 35.

per week—less than 1/70 of the average food intake of a 100 gm. rat on this food mixture.⁸ The outcome is shown in Chart 1.

In nearly every case there was a brief period of adjustment to the new and extremely unusual food mixture during which some loss of weight occurred. This often happens with various other artificial mixtures and is therefore not peculiar to the high protein regimen adopted. In the present series of feeding tests, as in others in which the food deviated markedly from the usual types of rations, an occasional animal succumbed—presumably through underfeeding—before the readjustment was made.

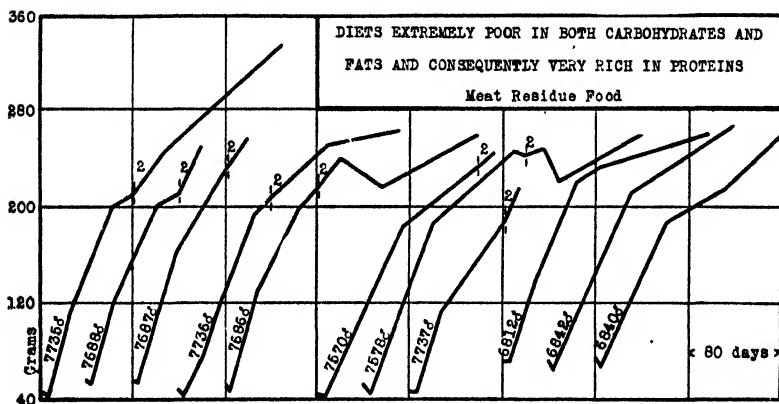


CHART 1.

The growth of Rat 7735 to a maximum of 336 gm. represents the largest degree of success attained with these extreme types of food mixture. As a rule the animals stopped gaining in weight at a maximum around 250 gm. The reason for this is not yet clear. The males from our stock colony and likewise many kept on more "normal" artificial mixtures of foods frequently attain a size above 300 gm. although 250 gm. represent good growth in many colonies. It is not an uncommon experience to find that our rats on experimental diets stop gaining before a body weight of 300 gm. is attained. The excellent rate of growth, while the

⁸ This calculation is based on statements regarding the content and digestibility of the carbohydrates in yeast and alfalfa hay as compiled by Henry and Morrison (Henry, W. A., and Morrison, F. B., *Feeds and feeding*, Madison, 18th edition, 1923).

gains continued, is worthy of emphasis. That the food is not an ideal ration for the approach to full adult size is suggested by the resumption of gains frequently observed when a male animal weighing less than 300 gm., which has stopped growing, is given a mixed diet on which stock rats thrive.

The favorable results just recorded with diets almost entirely devoid of preformed digestible carbohydrate, or at all events

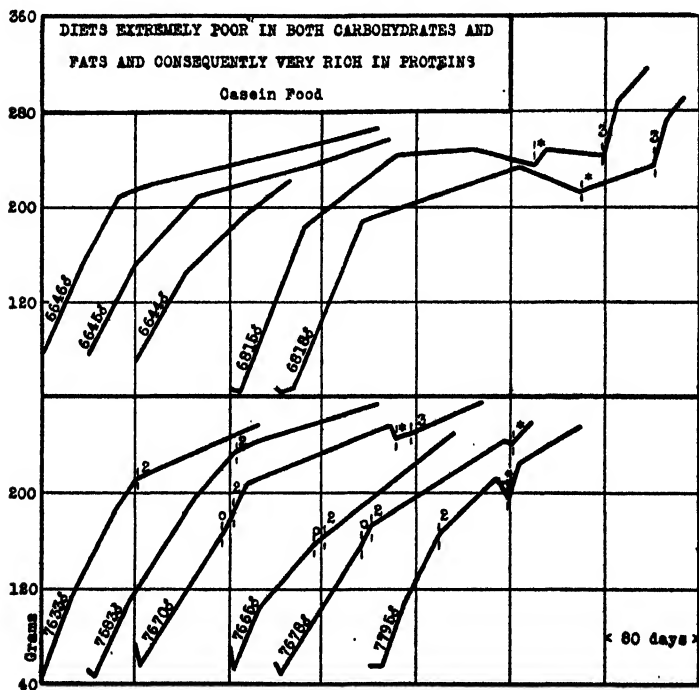


CHART 2.

extremely poor therein, are not restricted to meat alone as a source of protein. Noteworthy growth has been secured on diets in which casein furnished almost all the protein component. The casein was not highly purified and may have contained traces of lactose. The food mixtures consisted of casein 90 per cent, gliadin 5 per cent, salts 5 per cent, together with daily additions of yeast, 200 mg., and alfalfa, 400 mg. Zein replaced gliadin as an adhesive protein in the diets of Rats 6646, 6645,

6644, 6815, and 6818 (Chart 2). In Period 2 casein alone (95 per cent) was used as a source of protein. After the date indicated by "o" on the graphs, Rats 7670, 7665, and 7678 received daily 143 mg. of cod liver oil as an additional source of vitamine A. An asterisk (*) is inserted on a few of the graphs to indicate that the yeast supplement was increased to 400 mg. per day. The outcome is shown in Chart 2.

In the case of three animals, Rats 7670, 6815, and 6818, part of the protein was replaced in Period 3 with lard to the extent of 15 per cent of the food mixture. The change was followed by a gain in body weight for a time by two of these rats. This raised the question of the possible special rôle of fats in promoting gain at the end of the period of growth when the increments of weight may represent deposits in the adipose connective tissues rather than actual increments in the more active tissue cells. Since protein is notably not well adapted to the production of fat in animals it is conceivable that the gains above 250 gm. represent not true growth but "fattening" which is not secured readily from protein sources even though sugar is available as an intermediary product of its metabolism. It is noteworthy in this connection that in the tests with carbohydrate-poor rations in which some fat was incorporated the maximum body weight attained was in general greater than with the fat-free rations. This will be shown in records that follow.

Reference should be made here to some of the physiological effects observed in the animals on diets in which most of their energy was derived from protein. Diarrhea was rarely encountered despite the peculiarity of the food for the alimentary flora. The calorie intake of the fat-free, carbohydrate-free foods was not notably larger than that of rats fed with mixed "balanced" rations. The utilization must therefore have been adequate if not actually advantageous from the standpoint of energy metabolism. A conspicuous feature was the large intake of water and the very marked diuresis—an outcome to be expected where such unusually large relative quantities of urea and other protein catabolites must be disposed of through the kidneys. The marked enlargements of these organs will be discussed elsewhere.⁹

⁹ A preliminary account has been published by Osborne, Mendel, Park, and Darrow (Osborne, T. B., Mendel, L. B., Park, E. A., and Darrow, D., *Proc. Soc. Exp. Biol. and Med.*, 1922-23, xx, 452).

In somewhat comparable experiments which Drummond, Crowden, and Hill¹⁰ made with a diet consisting of casein 83 per cent, yeast extract 5 per cent, lemon juice 5 per cent, shark liver oil 2 per cent, salt mixture 5 per cent, the animals "maintained excellent health throughout" but the maximum body weight did not exceed 146 gm. at 130 days of age after feeding the rats on the high protein diet for 100 days. The recent report of Asada¹¹ is not in harmony with our experience.

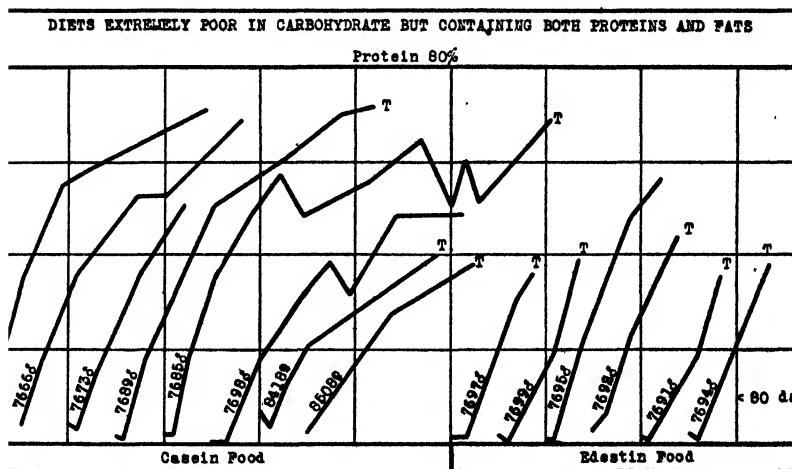


CHART 3.

Experiments with Diets Extremely Poor in Carbohydrate but Containing Both Proteins and Fats.

Feeding tests in which lard replaced part of the protein showed in general somewhat better growth, both as to the rate of gain and the maximum weight reached, than was secured with the food mixtures practically devoid of both fat and carbohydrate. The lowest proportion of fat tested was in diets consisting of protein (casein or edestin) 80 per cent, lard 15 per cent, salts 5 per cent, together with daily supplements of dried alfalfa, 400 mg., and

¹⁰ Drummond, J. C., Crowden, G. P., and Hill, E. L. G., *J. Physiol.*, 1922, lvi, 413.

¹¹ Asada, K., *Biochem. Z.*, 1923, cxxxix, 234.

dried brewery yeast, 200 mg. as sources of vitamins A and B, respectively. Chart 3 shows the results obtained up to the point of maximum weight.

Some of the experiments (marked "T") were terminated while the animals were still growing. The trials with edestin are particularly interesting because this protein, obtained by crystallization, could at most have furnished only minute traces, if any, of contaminating carbohydrate. Several attempts to raise animals on fat-free diets containing 95 per cent of protein of which 90 per cent was edestin failed because the animals died during the preliminary period of adjustment to the food.

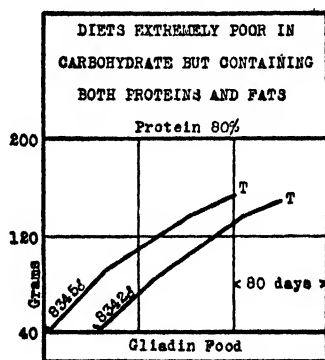


CHART 4.

Rats thrive even when gliadin supplied most of the nitrogenous intake. The low yield of the essential amino acid lysine from this protein is a limiting factor in growth, as has been shown in earlier studies by us.¹² Rats 8342 and 8345 (Chart 4) received a ration containing gliadin 80 per cent, salts 5 per cent, lard 12 per cent, cod liver oil 3 per cent, supplemented daily by 80 mg. of the Osborne-Wakeman yeast fraction II diluted with 120 mg. of casein. Owing to the large consumption of gliadin on this high protein diet, and possibly also the small supplement represented by 120 mg. of casein per day, enough lysine was liberated

¹² Osborne, T. B., and Mendel, L. B., Feeding experiments with isolated food-substances, *Carnegie Inst. Washington, Pub. 156*, pt. 2, 1911; *Z. physiol. Chem.*, 1912, lxxx, 307; *J. Biol. Chem.*, 1912, xii, 473; 1914, xvii, 325; 1916, xxv, 1.

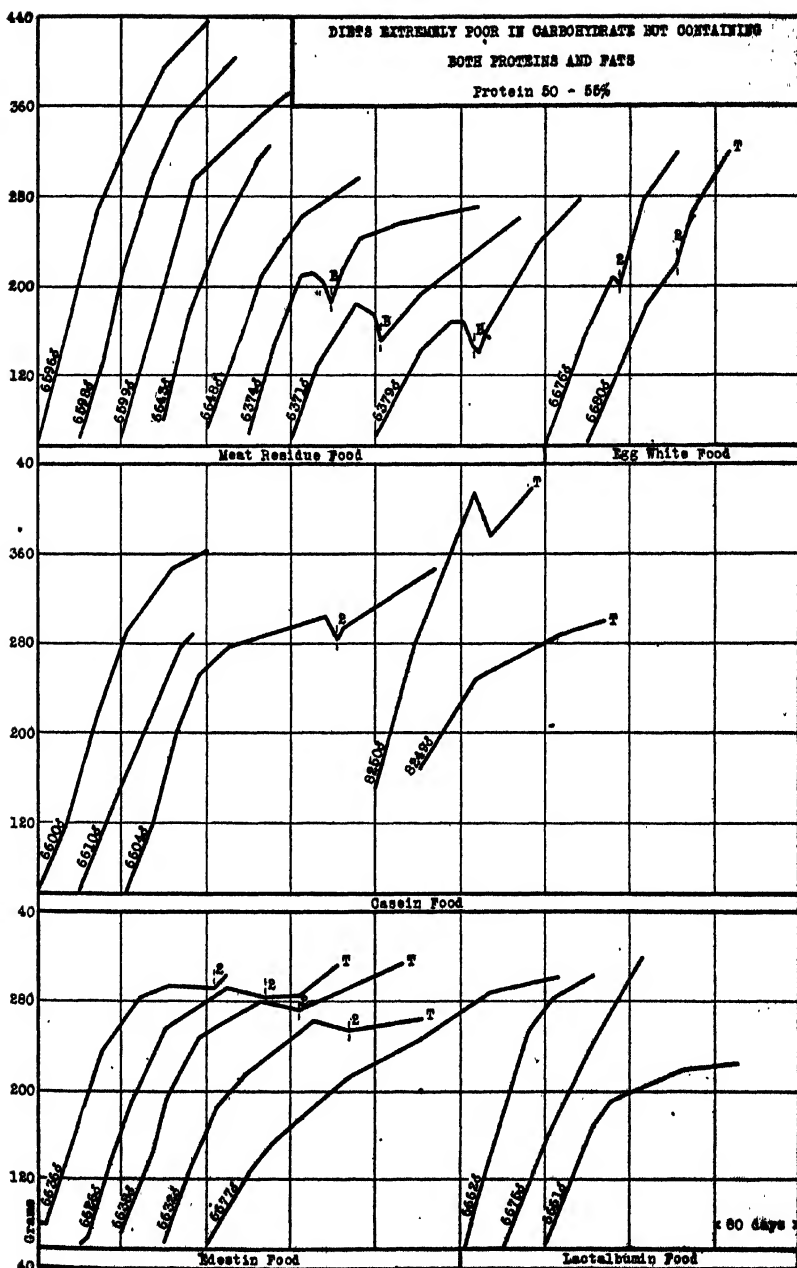


CHART 5.

to permit considerable growth. In these experiments the intake of preformed carbohydrate was reduced to an extreme minimum represented by the small amount possibly present in the yeast fraction which gave no reaction with Fehling's test even after hydrolysis. The extent and rate of gains made are shown in Chart 4.

The outcome of the tests with diets deficient in preformed carbohydrate was surprisingly good even when fat comprised

TABLE I.
Diets of Rats Shown in Chart 5.

Food ingredients.	Meat residue protein.			Casein.		Edestin.		Lactalbumin.	Egg white.
	Rats 6596 6598 6599	Rats 6643 6648	Rats 6374* 6371* 6379*	Rats 6800 6810 6804†	Rats 8250 8249	Rats 6636† 6626† 6638† 6632†	Rat 6677	Rats 6662 6675 6661	Rats 6676† 6680†
	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
Meat residue.....	50	55	50						
Casein.....				50	55				
Edestin.....						50	55		
Lactalbumin.....								55	
Egg white.....									55
Lard.....	31	31	40	31	37	31	31	31	31
Butter fat.....	9	9		9		9	9	9	9
Cod liver oil.....					3				
Agar.....	5		5	5		5			
Salt mixture.....	5	5	5	5	5	5	5	5	5

Daily supply of vitamine B: yeast 400 mg.

* In the period beginning at B part of the lard was replaced by butter fat (9 per cent).

† In Period 2 meat residue replaced the protein previously fed.

‡ In Period 2 casein replaced the egg white previously fed.

nearly two-thirds of the calorie intake and protein little more than one-third. The need of endogenous sugar, whereby the fats could "burn in the flame of the carbohydrate," must have been large in such instances; yet sugar was evidently made available, if it is indeed requisite for this purpose. Success was attained with the five different proteins tested, in food mixtures of approximately the following composition: protein 50 to 55 per cent; salts 5 per cent; agar 0 to 5 per cent; lard 31 to 40 per

cent; butter fat 0 to 9 per cent; cod liver oil 0 to 3 per cent; together with a daily supplement of dried yeast, 400 mg. The details of the rations for the rats referred to in Chart 5 are given in Table I.

In some of the earlier tests agar was used in the ration to supply "roughage." The objection may be made that this polysaccharide material, though presumably entirely indigestible,¹⁸ might afford a small amount of soluble assimilable carbohydrate through bacterial or other reactions. This rather improbable assumption loses its significance in view of the fact that a considerable number of the animals grew satisfactorily in the absence of agar from the diet (Rats 6643 and 6648 on meat residue food, Rat 6677 on edestin food, and Rats 8250 and 8249 on casein food, as well as all the rats on the lactalbumin and egg white foods). It is interesting to note that though Rats 6371, 6374, and 6379 on the meat residue food received practically no vitamine A and showed the characteristic nutritive disaster in due time, they recovered and grew when this vitamine was given in the form of butter fat during the period beginning at B—the recovery taking place despite the lack of any preformed carbohydrate in a diet that furnished considerably more than one-half of its energy in the form of fat.

Experiments with Diets Entirely Devoid of Preformed Carbohydrate.

Most of the experiments thus far recorded are open to the criticism that the food furnished *some* preformed carbohydrate, small though it may have been. One thinks of the possibility of glycogen in the meat residue, of lactose adherent to the casein and lactalbumin prepared from milk. Yeast is known to include small quantities of glycogen at times. To eliminate even these essentially negligible sources of error and approach as nearly as present day experimental requirements will permit to an absolutely carbohydrate-free ration, we have conducted additional feeding trials with preparations of casein, edestin, and lactalbumin, none of which gave the Molisch reaction for carbohydrates.

The yeast fraction used, even after hydrolysis with acid, gave no reduction with Fehling's solution. Furthermore, the quan-

¹⁸ Swartz, M. D., *Tr. Connecticut Acad. Arts and Sc.*, 1911, xvi, 247.

in as far as carbohydrate is required for the intermediary metabolism of fats it can be furnished endogenously throughout the period of growth to adult size, even when more than half of the energy intake is supplied in the form of fat.

Experiments with Diets Extremely Poor in Carbohydrate but Containing Protein and Fatty Acids.

In current discussions regarding the ketogenic-antiketogenic balance in man it has been assumed¹⁴ that not only amino acids derived from protein but also glycerol derived from fats are con-

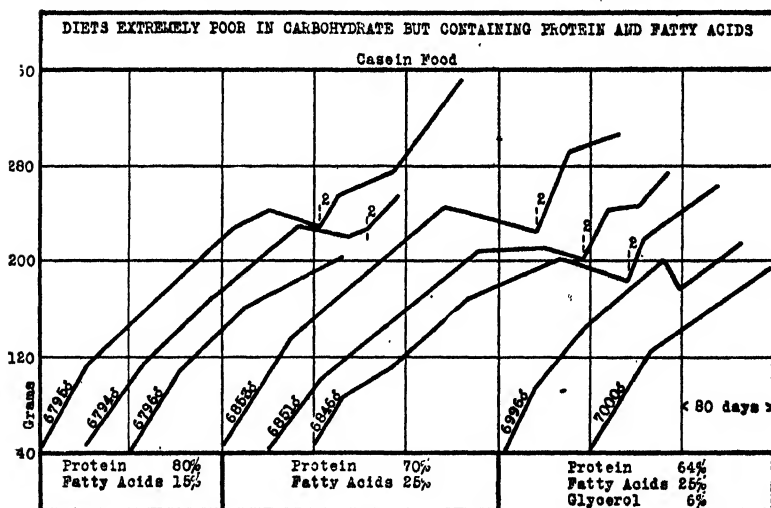


CHART 7.

verted into glucose, thus exerting a ketolytic action. This assumption lends interest to feeding tests which we have conducted with mixtures of casein, fatty acids, and salts, along with small supplements of alfalfa meal and yeast to supply vitamins A and B. The mixed fatty acids used, which were prepared from lard, were carefully washed with water. Rats 6996 and 7000 (Chart 7) received glycerol in place of part of the protein. The food mixtures of Period 1 for the animals referred to in Chart 7 were constituted as shown in Table II.

¹⁴ See Shaffer, P. A., *J. Biol. Chem.*, 1922, liv, 400.

In Period 2 lard replaced the fatty acids in the mixture. The rate of growth, it will be observed, was scarcely modified at all by the inclusion of glycerol in the food mixtures.

*Experiments with Diets Extremely Poor in Carbohydrate,
with Low Protein and High Fat Content.*

Diets not only devoid of preformed carbohydrate but offering the energy largely in the form of fats, with relatively less protein, present a unique problem of nutritive possibilities from the standpoint of the ketogenic-antiketogenic balance of the body, in as far as the supply of potential sources of sugar are concerned. With such mixtures the fact that the food intake is practically limited by the energy needs of the animal leads to sometimes unanticipated limitations that deserve greater emphasis than

TABLE II.
Diets of Rats Shown in Chart 7.

Food ingredients.	Rats 6795, 6794, and 6796.	Rats 6853, 6851, and 6846.	Rats 6996 and 7000.
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Casein.....	80	70	64
Fatty acids.....	15	25	25
Glycerol.....			6
Salt mixture.....	5	5	5

Daily supply of vitamins A and B: dried alfalfa 400 mg., dried yeast 200 mg.

has been given to them in the past. When two foods contain the same *percentage* of protein or salts, for example, but different proportions of fat, the total food intake will vary in accord with the relative energy yield. Less of the food rich in fat will furnish the same amount of energy as a larger quantity of the mixture poorer in fat. Consequently, the *absolute* intake of protein or salts will be smaller on the fat-rich regimen. A limitation of growth or maintenance may thus result because the minimum requirement for certain nitrogenous or inorganic components is no longer satisfied. For this reason we have not succeeded in securing growth on diets consisting of more than three-quarters of fat; *i.e.*, where more than 8/10 to 9/10 of the calorie intake was represented by fat.

The most successful outcome with practically carbohydrate-free diets very rich in fat was secured with a food mixture consisting of meat residue 20 per cent, beef fat 61 per cent, butter fat 14 per cent (total fat 75 per cent), salt mixture 5 per cent, vitamine B being furnished each day by tablets containing a mixture of Osborne-Wakeman yeast fraction II (2 parts) and casein (3 parts). The growth records are shown in Chart 8, Rats 6970 and 6968. During Period 1 the rats received daily 40 mg. of the yeast fraction and 60 mg. of casein; in Period 2 this quantity was doubled, thereby furnishing to each rat 0.84 gm. of casein per week in addition to the meat protein consumed.

A second series of animals—Rats 6690, 6694, and 6688, all females—showed increments of body weight equally good for this less rapidly growing sex. The food of these rats contained meat residue 30 per cent, beef fat 56 per cent, butter fat 9 per cent (total fat 65 per cent), salt mixture 5 per cent, the vitamine B supplement in Periods 1 and 2, respectively, being like that of Rats 6970 and 6968. During the latter part of the tests more butter fat was introduced at "B" and cod liver oil added at "C." In every case, however, the ration retained its essentially carbohydrate-free character and its exceptional richness in fats.

To what extent we succeeded in duplicating these results by the use of casein in the diet is shown by the records of two groups of rats: Nos. 6746 and 6777 and 7465, 7396, and 7377, in Chart 8. Except as later specified their rations had the composition shown in Table III.

These feeding trials were not conducted in the usual uniform way because they were pioneer tests in character, and changes in the feeding scheme were made from time to time, as follows:

- | | |
|------------------------------|--|
| Rats 6746 and 6777 at | 2 Vitamine B supplement doubled. |
| | C 143 mg. cod liver oil given daily. |
| Rats 7465, 7396, and 7377 at | D 0.8 gm. casein given daily. |
| | E 0.4 gm. yeast replaced yeast fraction. |
| | F 0.5 per cent cystine added. |
| | G Cystine omitted. |
| | H 0.2 gm. yeast replaced yeast fraction. |
| | I Yeast doubled. |

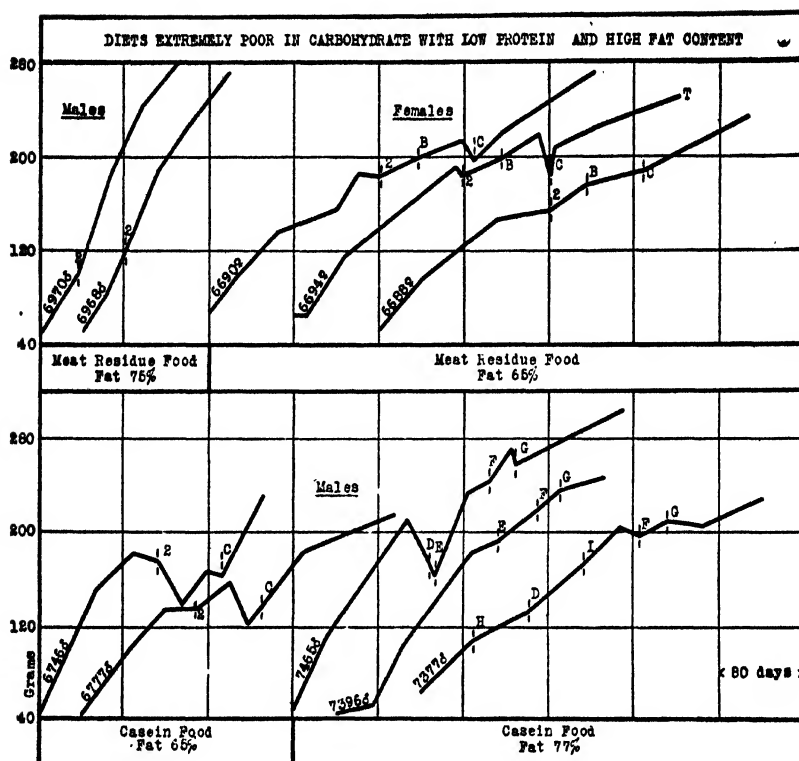


CHART 8.

TABLE III.
Diets of Rats Shown in Chart 8.

Food ingredients.	Rats 6746 and 6777.	Rats 7465, 7396, and 7377.
	per cent	per cent
Casein.....	30	18
Salt mixture:.....	5	5
Lard.....	56	61
Butter fat.....	9	14
Cod liver oil.....		2
Total fats.....	65	77
Daily supply of vitamine B.		
{ Yeast fraction.....	40 mg.	80 mg.
{ Casein.....	60 "	120 "

All our attempts to feed mixtures containing as little as 15 per cent of casein with 80 per cent of fat along with salts 5 per cent and vitamine B supplements failed. In interpreting the outcome of the experiments with Rats 7465, 7396, and 7377 it must be remembered that several of the changes made in the regimen (such as use of yeast and additions of casein) incidentally altered the proportionate relationship between the proteins and fats ingested. The inclusion of cystine was made in order to avert any failure to grow possibly due to the lack of enough of this amino acid at low levels of protein intake, such as we¹⁵ have found in other studies.

Glycogen Formation on Carbohydrate-Free Diets.

That glycogen can be formed in the body from protein has long been an accepted fact in physiology.¹⁶ The directly available experimental data have been secured by feeding tests of brief duration usually following a period of starvation intended to deplete the body store. There is the remote though improbable possibility that after a prolonged carbohydrate-free regimen the surplus of sugar in the metabolism would be too small to permit any noteworthy storage of glycogen. Estimations made by C. S. Leavenworth of the glycogen content of the entire bodies of three rats (Nos. 6636, 6662, and 6675, Chart 5), which had received diets devoid of carbohydrate, showed a content of 0.271 gm. per animal or 0.09 per cent of the body weight; for control animals on the usual starch-containing food mixtures the figures were 0.366 gm. or 0.12 per cent.

The Ketogenic-Antiketogenic Balance.

The estimation of the ratio between the ketogenic and anti-ketogenic factors at which ketosis is averted has been the subject of considerable debate, particularly in its relation to human diabetics. Ladd and Palmer¹⁷ have recently proposed a simplified formula for comparing the fat and available carbohydrate,

¹⁵ Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1915, xx, 351.
Mendel, L. B., *J. Am. Med. Assn.*, 1915, lxiv, 1539.

¹⁶ Cremer, M., *Ergebn. Physiol.*, 1902, i, 803.

¹⁷ Ladd, W. S., and Palmer, W. W., *Am. J. Med. Sc.*, 1923, clxvi, 157.

involving the ratio $\frac{\text{gm. fat}}{(0.58 \times \text{gm. protein}) + \text{gm. carbohydrate}}$, the data being derived from the composition of the diet. This formula takes no account of the antiketogenic glycerol derived from fats or of ketogenic derivatives of the protein. Clinical experience has shown, however, that when the ratio exceeds 4:1, ketosis invariably ensues. A similar calculation for our rats (Chart 8, Rats 6970, 6968, 7465, 7396, and 7377) on diets comparatively poor in protein (about 20 per cent) and very rich in fat (about 75 per cent) indicates a ratio of over 7:1. The other dietaries on which growth was secured show ratios within the 4:1 limit proposed by Ladd and Palmer.

From a physiological standpoint the behavior of the animals on the carbohydrate-free rations is of further interest in the light of the newer studies regarding the so called isodynamic replacement of nutrients in the energy metabolism. Careful experiments by Krogh and Lindhard¹⁸ have shown that in the transformation of energy incident to muscle work fat and carbohydrate by no means function in isodynamic proportions. Comparable tasks may require as much as 10 per cent more energy when it is liberated at the expense of fats. This diminished efficiency is said to be attended with an earlier onset of fatigue. Likewise, Cathcart¹⁹ found that isodynamic quantities of fat and glucose are not equivalent in protecting the body from loss of protein and in determining the types of nitrogenous metabolism.

SUMMARY.

The reputed rôle of carbohydrate in the metabolism of matter and energy in the body is discussed, particular reference being made to the so called ketolytic or antiketogenic function of sugar and also to the profound untoward effects of hypoglycemia.

An outline is given of the methods employed in nutrition experiments in which the amount of available *preformed* carbohydrate was so small as to be undetectable. The food contained various proteins and fats in widely different proportions, along with a suitable inorganic salt mixture and suitable sources of vitamins A and B.

¹⁸ Krogh, A., and Lindhard, J., *Biochem. J.*, 1920, xiv, 290.

¹⁹ Cathcart, E. P., *Biochem. J.*, 1922, xvi, 747.

Some of the food mixtures furnished the energy essentially in the form of proteins—"meat residue" or casein; good growth was secured in many trials, although as a rule the male rats stopped gaining in weight at a maximum around 250 gm. Further gains were often secured by change to a "mixed" diet, or by addition of some fat. It is suggested that perhaps the later gains represent "fattening" rather than true growth. Diuresis and hypertrophy of the kidneys were often noted, although there was no evidence of damage to these organs. The average weight of the kidneys was almost twice that of the kidneys of control animals and their size was about one-third greater. Microscopic examination made by Professor Park and his colleagues failed to disclose any changes of an inflammatory or degenerative nature. In this respect our findings seem to be at variance with those recently reported by Polvogt, McCollum, and Simmonds,²⁰ who have described serious lesions of the kidney in rats subsisting on diets which, though termed "excessive" in protein, contained a considerably lower content than the rations in our present experiments. This raises the question as to the reputed rôle of "high protein" feeding in renal disorder as claimed by Newburgh²¹ and others. The intake of food on the protein diets free from carbohydrate and fat was not notably large, thus indicating that "protein calories" are well used in the metabolism of energy.

In numerous experiments involving foods containing different proteins—"meat residue," casein, edestin, egg albumin, and lactalbumin—and containing varying proportions of fats along with the other dietary essentials but without preformed carbohydrate, good growth was secured in many instances to large size. Even when a protein as "abnormal" in its amino acid make-up as is gliadin was used in large proportions some growth was secured. In the three rats examined the glycogen content of the body was comparable to that of control animals on standard, starch-containing food. In many of the experiments there was an entire absence of "roughage" to produce bulk in the feces.

The absence of preformed carbohydrate did not prevent or

²⁰ Polvogt, L. M., McCollum, E. V., and Simmonds, N., *Bull. Johns Hopkins Hosp.*, 1923, xxxiv, 168.

²¹ Newburgh, L. H., *Medicine*, 1923, ii, 77.

alter the prompt recovery of rats which had previously declined on foods containing no vitamine A when this factor was again supplied.

The ability of animals to grow on diets in which the organic foods consisted essentially of protein and fatty acids is demonstrated. The presence of glycerol did not produce any noteworthy advantage.

Estimation of the ketogenic-antiketogenic ratio of the foods according to Ladd and Palmer's formula

$$\frac{\text{gm. fat}}{(0.58 \times \text{gm. protein}) + \text{gm. carbohydrate}}$$

gives in most instances figures less than 4:1, the limit beyond which ketosis is said to arise in man. In some of the experiments with foods extremely rich in fats, without apparent detriment to the animals, this ratio was greatly exceeded.

Our experiments indicate that *in as far as carbohydrate is required for the intermediary metabolism, particularly for the metabolism of fats and the development of energy in muscular contraction, it can be furnished endogenously throughout the period of growth to adult size.*

CELL VOLUME AND ELECTRICAL CONDUCTIVITY OF BLOOD.

By H. C. GRAM.*

(From the John Herr Musser Department of Research Medicine, University of Pennsylvania, Philadelphia.)

(Received for publication, December 21, 1923.)

In a former paper with Norgaard (1) we dealt with the conductivity of whole blood and plasma and the calculation of the cell volume from these data. We assumed at first that the corpuscles had no conductivity and also no influence excepting the decrease of the conducting medium. Calculating in this way we found that the sodium chloride equivalent (or K_{20}) would not give the true cell volume, while this could be calculated fairly accurately by using the scale values of our ionometer. Since this was an accidental property of the individual ionometer used, we left out this part of the work in the English translation (2) of our paper. The surmise that the corpuscles, though non-conducting, had no special influence except that of lessening the amount of conducting medium evidently was not true, as may be seen from Maxwell's (3) formula¹ for conductivity of suspensions or from the work of Oker-Blom (4) and others. As the question was still somewhat doubtful and several conflicting formulas exist (Stewart, 5; Bugarszky and Tangl, 6) I resolved to determine cell volume by the hematocrit and the conductivity of whole blood and serum both by the ionometer and the Wheatstone bridge. The formula

* Robert M. Girvin Fellow in Research Medicine and Fellow of The Rockefeller Foundation. Aided by a grant from the Edward N. Gibbs Fund of the New York Academy of Medicine.

¹ Simplified and restated for our purposes this would be

$$\text{Cell volume} = \frac{100 (2K_s - 2K_b)}{K_b + 2K_s}$$

K_b = conductivity of whole blood.

K_s = conductivity of serum.

S.H. M. Cholelith?	As drawn.	Sept. 20	46.1	0.662	108.1	0.653	106.7	0.240	41.8	0.238	41.4	2.58 2.59	47.0 47.1
E.G. F.	Concentrated.	Nov. 1	45.6	0.655	107.0	0.651	106.4	0.245	42.6	0.248	43.1	2.47 2.51	45.3 46.0
Dog 22. M.	As drawn.	Oct. 8	45.4	0.700	114.1	0.701	114.4	0.260	45.0	0.253	43.9	2.61 2.54	47.4 46.4
D.H.M. M. Normal.	Diluted with own serum.	Oct. 20	43.7	0.678	110.6	0.669	109.2	0.279	48.0	0.271	46.8	2.34 2.30	43.2 42.5
E.G. F. Normal.	As drawn.	Nov. 1	38.4	0.655	107.0	0.651	106.4	0.295	50.5	0.302	51.8	2.05 2.12	37.6 39.0
D.H.M. M. Normal.	Diluted with own serum.	Oct. 20	36.4	0.678	110.6	0.669	109.2	0.323	55.0	0.321	54.5	2.00 2.01	36.3 36.7
Dog 23. M.	Diluted with own serum.	Sept. 22	33.7	0.732	119.1	0.719	117.0	0.359	60.9	0.360	61.0	1.92 1.96	34.6 35.5
B. M. Pneumonia.	As drawn.	Sept. 26	28.8	0.582	96.0	0.580	95.6	0.323	55.0	0.316	54.0	1.77 1.75	30.7 30.1

* A curve drawn through these points in coordination with the corresponding ratios will allow an approximate determination of cell volume.

TABLE I—Concluded.

Name. Sex. Diagnosis.	Blood.	Date.	Cell volume (hematocrit).	Serum.				Blood.				Ratio $\frac{K_2}{K_1}$ (1) (2) Ionom- eter.	Cell volume by own curve drawn from these obser- va- tions*
				Ionometer.		Bridge.		Ionometer.		Bridge.			
				NaCl equiv- alent.	K_{20} $\times 10^4$	NaCl equiv- alent.	K_{20} $\times 10^4$	NaCl equiv- alent.	K_{20} $\times 10^4$	NaCl equiv- alent.	K_{20} $\times 10^4$		
				per cent		per cent		per cent		per cent			
J.H.A. M. Normal.	Diluted with own serum.	1923 Oct. 2	28.5	0.684	111.5	0.674	110.0	0.396	66.6	0.389	65.5	1.68 1.67	28.0 27.7
J.H.A. M. Normal.	Diluted with own serum.	Oct. 2	26.4	0.684	111.5	0.674	110.0	0.413	69.4	0.410	69.0	1.59 1.61	25.2 25.8
S.H. M. Cholelith.	Diluted with NaCl solution.	Sept. 20	13.6	0.960	149.8	0.962	150.5	0.740	120.3	0.737	119.9	1.26 1.25	13.9 13.6

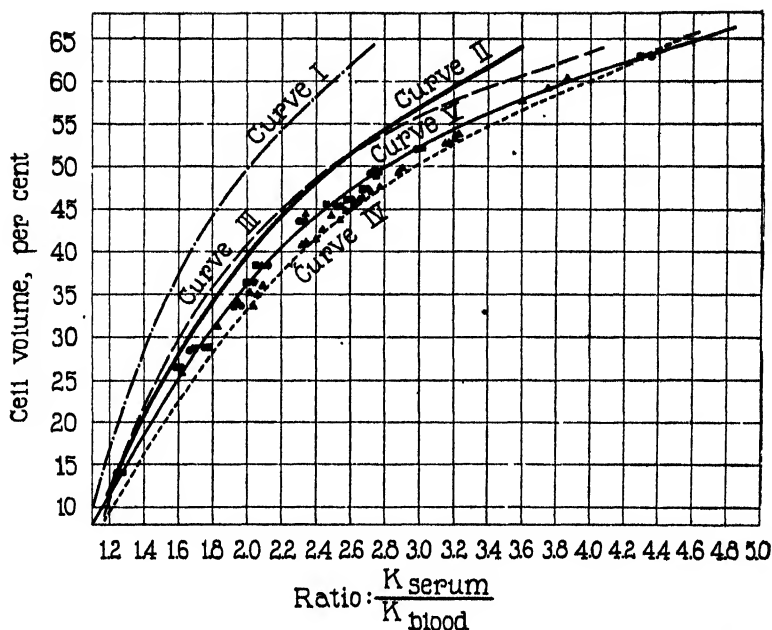


FIG. 1. Curve I drawn by assuming the corpuscle conductivity = 0 and no further interference by presence of corpuscles than that caused by displacement of serum.

Curve II drawn by Maxwell's formula assuming the cell conductivity = 0.

$$\left[\text{Cell vol. per cent} = \frac{100 (2K_s - 2K_b)}{K_b + 2K_s} \right]$$

Curve III drawn by Bugarszky and Tangl's formula: $100 - \text{cell volume} = 92 \frac{K_b}{K_s} + 13$, solving this for $\frac{K_s}{K_b}$ and introducing the various cell volumes.

Curve IV, Fraenckel's empirical curve for the relation $\frac{K_s}{K_b}$ and cell volume.

Curve V, the author's empirical curve for the relation $\frac{K_s}{K_b}$ and cell volume.

■ represents the author's determinations by Wheatstone bridge; ●, author's determinations by ionometer; and ▲, Stewart's results. (Ratio $\frac{K_s}{K_b}$ obtained from Stewart's values. Cell volumes calculated by him from his formula $100 - \text{vol. per cent} = \frac{\lambda_b}{\lambda_s} (180 - \lambda_b - \sqrt{\lambda_b})$.)

of Maxwell apparently only holds good when the cell volume is very small, so that for simplicity's sake I adopted the graphic registration of the ratio $\frac{\text{conductivity of serum}}{\text{conductivity of whole blood}}$ as suggested by Oker-Blom (4) and Fraenckel (7). The blood used was defibrinated blood of normal and anemic men and dogs, which sometimes was concentrated by withdrawing some serum, sometimes diluted by serum or in one case by salt solution.

TABLE II.

$K_{20} \times 10^4$	NaCl equivalent.
	<i>per cent</i>
20	0.112
25	0.141
30	0.170
35	0.199
40	0.229
45	0.260
50	0.291
55	0.323
60	0.354
65	0.385
70	0.417
75	0.449
80	0.480
85	0.512
90	0.544
95	0.576
100	0.609
105	0.641
110	0.674
115	0.706
120	0.738

The results as given in Table I and in Fig. 1 show that this method of estimating the cell volume is feasible for the blood of both men and dogs, and whether the salt content of the serum is high or low. The ionometer results were quite as good as those obtained by the more difficult bridge determination. This allows a quick estimation of the cell volume. The procedure is to divide the conductivity (K_{20}) of serum by that of whole blood and read the cell volume corresponding to the observed ratio from a curve

prepared by coordinating the corresponding values in the last two columns of Table I. In order to translate the NaCl equivalents into K_{20} the following corresponding values are given in Table II.

Fig. 1 also shows that the presence of evenly suspended corpuscles does more than merely replace the conducting liquid between the electrodes (Curve I). Maxwell derived a formula which expresses the effect of bodies in suspension on conductivity (Curve II), but as pointed out by Maxwell his formula does not hold at high suspension volume percentages such as are met with in blood. Bugarszky and Tangl's empirical formula gives a curve which is considerably at variance with ours (Curve III).

The cell volumes calculated by Stewart's empirical formula on Stewart's own data coordinated with the conductivity ratio calculated from his data agree fairly well with our empirical curve (Curve V) and with that of Fraenckel (Curve IV); the majority falling between these two curves. Though Stewart's formula, being based on determinations at 5°C., cannot without change of constants be applied to our observations, the plotting of his data in comparison with ours shows that the relation between the ratio $K_s : K_b$ and cell volume is valid over a wide range of temperature. Direct hematocrit measurement of cell volume (with transparency at the end-point) as used in this study is probably a more accurate method for cell volume than the colorimetric determination of added hemoglobin in serum as used by Stewart in deriving his empirical cell volume-conductivity formula and than Bleibtreu's dilution method used by Fraenckel in preparing his empirical curve.

SUMMARY.

1. The ionometer and bridge values of conductivity of serum and of whole blood at various cell volumes have been determined and an empirical curve coordinating the ratio $K_{\text{serum}} : K_{\text{blood}}$ and cell volume has been drawn.
2. These results have been compared with those of previous workers on the subject.

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ANIMAL CALORIMETRY.

TWENTY-FOURTH PAPER.

ANALYSIS OF THE OXIDATION OF MIXTURES OF CARBOHY- DRATE AND FAT,

A CORRECTION.

BY GRAHAM LUSK.

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(Received for publication, December 10, 1923.)

In 1901 Zuntz and Schumburg¹ published a standard table showing the caloric value of a liter of oxygen when used to oxidize mixtures of carbohydrate and fat. Figures were given for respiratory quotients varying from 1.00, for pure carbohydrate, to 0.707, for pure fat. The writer elaborated this table² and introduced columns showing the relative quantity in calories of carbohydrate and fat consumed. That these figures were in error was pointed out to me by Dr. H. H. Mitchell in a letter dated June 26, 1917, and the error has since been called to my attention by my students. To one of them, Mr. A. M. Michaelis, I am indebted for the following corrected table. Although the error is not great, yet it is worthy of note and of record. The table serves to illuminate the charts recently prepared by Dr. E. F. Du Bois, which he presents in the article immediately following this.

¹ Zuntz, N., and Schumburg, *Studien zu einer Physiologie des Marsches*, Berlin, 1901, 361.

² Williams, H. B., Riche, J. A., and Lusk, G., *J. Biol. Chem.*, 1912, xii, 357. Lusk, G., *The elements of the science of nutrition*, Philadelphia, 3rd edition, 1917, 61.

R. Q.	Percentage of total oxygen consumed by:		Percentage of total heat produced by:		Calories per liter O ₂ .	
	Carbo-hydrate. (1)	Fat. (2)	Carbo-hydrate. (3)	Fat. (4)	Number. (5)	Logarithm. (6)
0.707	0	100.0	0	100.0	4.686	0.67080
0.71	1.02	99.0	1.10	98.9	4.690	0.67114
0.72	4.44	95.6	4.76	95.2	4.702	0.67228
0.73	7.85	92.2	8.40	91.6	4.714	0.67342
0.74	11.3	88.7	12.0	88.0	4.727	0.67456
0.75	14.7	85.3	15.6	84.4	4.739	0.67569
0.76	18.1	81.9	19.2	80.8	4.751	0.67682
0.77	21.5	78.5	22.8	77.2	4.764	0.67794
0.78	24.9	75.1	26.3	73.7	4.776	0.67906
0.79	28.3	71.7	29.9	70.1	4.788	0.68018
0.80	31.7	68.3	33.4	66.6	4.801	0.68129
0.81	35.2	64.8	36.9	63.1	4.813	0.68241
0.82	38.6	61.4	40.3	59.7	4.825	0.68352
0.83	42.0	58.0	43.8	56.2	4.838	0.68463
0.84	45.4	54.6	47.2	52.8	4.850	0.68573
0.85	48.8	51.2	50.7	49.3	4.862	0.68683
0.86	52.2	47.8	54.1	45.9	4.875	0.68793
0.87	55.6	44.4	57.5	42.5	4.887	0.68903
0.88	59.0	41.0	60.8	39.2	4.899	0.69012
0.89	62.5	37.5	64.2	35.8	4.911	0.69121
0.90	65.9	34.1	67.5	32.5	4.924	0.69230
0.91	69.3	30.7	70.8	29.2	4.936	0.69339
0.92	72.7	27.3	74.1	25.9	4.948	0.69447
0.93	76.1	23.9	77.4	22.6	4.961	0.69555
0.94	79.5	20.5	80.7	19.3	4.973	0.69663
0.95	82.9	17.1	84.0	16.0	4.985	0.69770
0.96	86.3	13.7	87.2	12.8	4.998	0.69877
0.97	89.8	10.2	90.4	9.58	5.010	0.69984
0.98	93.2	6.83	93.6	6.37	5.022	0.70091
0.99	96.6	3.41	96.8	3.18	5.035	0.70197
1.00	100.0	0	100.0	0	5.047	0.70303

Formula for
Column

(R. Q. = R)

- (1) % = $100 \frac{R - 0.707}{0.293}$
- (2) % = $100 \frac{1.00 - R}{0.293}$
- (3) % = $\frac{504.7 (R - 0.707)}{5.047 (R - 0.707) + 4.686 (1.00 - R)}$
- (4) % = $\frac{468.6 (1.00 - R)}{5.047 (R - 0.707) + 4.686 (1.00 - R)}$
- (5) Calories = $4.686 + \frac{R - 0.707}{0.293} \times 0.361$
- (6) Logarithm = log of Column 5

CLINICAL CALORIMETRY.

THIRTY-FIFTH PAPER.

A GRAPHIC REPRESENTATION OF THE RESPIRATORY QUOTIENT AND THE PERCENTAGE OF CALORIES FROM PROTEIN, FAT, AND CARBOHYDRATE.

By EUGENE F. DU BOIS.

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(Cornell) Medical Division, Bellevue Hospital, New York City.)*

(Received for publication, December 10, 1923.)

In the human metabolism there are always three factors: protein, fat, and carbohydrate. If, therefore, we wish to represent graphically the changes which may occur, it seems best to employ a triangle with each corner representing one of these foodstuffs. Irving Fisher (1) has used a right-angled triangle to show the composition of foods and diets. For metabolic mixtures we may draw a triangle which shows the respiratory quotient as well as the percentages of calories.

Lusk (2) has summarized the calculations of Loewy and has given the method for estimating the total metabolism and the percentage of calories furnished by carbohydrate, fat, and protein, using the following figures.

	R. Q.	Calorific value of 1 liter of oxygen.
Fat.....	0.707	4.686
Protein.....	0.801	4.485
Starch.....	1.000	5.047

In the preceding article Lusk (3) gives the formulas on which the calculations are based and makes a slight correction in the relative percentages furnished by fat and carbohydrate. These formulas served to establish the points on the base lines of the triangles here presented.

In each triangle the lower left-hand corner, at the respiratory quotient of 0.707, represents the theoretical point at which 100 per cent of the calories are derived from fat. The lower right-hand corner, over the respiratory quotient of 1.00, represents the point, never quite attained, where all the calories would be derived from carbohydrate. The peak, which is never even approached in man, is set at the point where 100 per cent of the calories would be derived from protein. This peak is, of course, placed directly over the respiratory quotient of 0.801. The subdivisions on the base line, as we have said before, are made according to the formulas given by Lusk. The subdivisions on the side lines are made according to similar formulas, using the appropriate factors for protein. In Fig. 1 these subdivisions are unevenly spaced on account of the nature of the formula.¹

By means of Fig. 1 we can rapidly find the percentage of calories furnished by carbohydrate if we know the total respiratory quotient and the percentage of calories furnished by protein.

The abscissæ represent the total respiratory quotient; the ordinates on the left-hand side of the chart, the percentage of calories from protein. The percentage from carbohydrate is determined from the diagonal lines with the figures on the right side of the triangle. Adding the percentages from protein and carbohydrate, we can subtract this sum from 100 and find the percentage from fat. For example, if the respiratory quotient were 0.90 and protein furnished 20 per cent of the calories, we can see that carbohydrate furnished 61 per cent and fat 19 per cent.

The accuracy of this chart has been tested by the data from many calorimeter experiments calculated according to the method of Zuntz and Schumburg, as given by Lusk (2), using the correction given in the preceding article. In no case was the divergence for

¹ I wish to thank Mr. A. M. Michaelis for his aid in checking my calculations. He has called my attention to the fact that the lines in this triangle should theoretically show curves which, however, are so slight that they may be disregarded. In the following paper of this series he gives two diagrams of a slightly more complicated construction. One of them is of great help in determining the total calories, the other may be used to correct the slight errors which have been published in the previous papers on "Clinical calorimetry" as a result of employing the uncorrected chart referred to by Lusk (3).

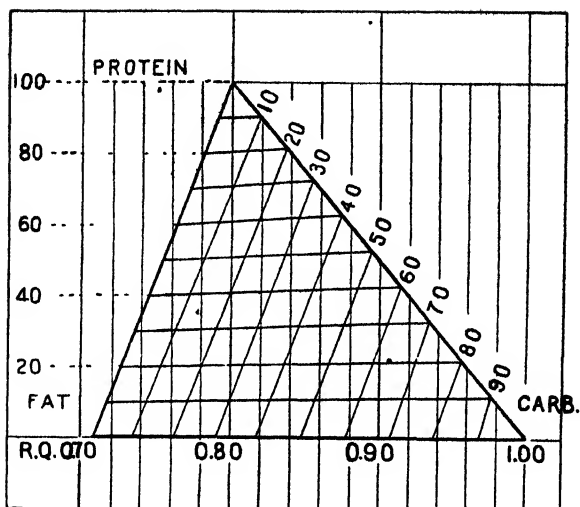


FIG. 1. Diagram showing the percentages of calories derived from protein, fat, and carbohydrate according to the respiratory quotient. The base line gives the total respiratory quotient; the ordinates reading on the left-hand side give the percentage of calories from protein; the diagonals reading on the right of the triangle give the percentage from carbohydrate.

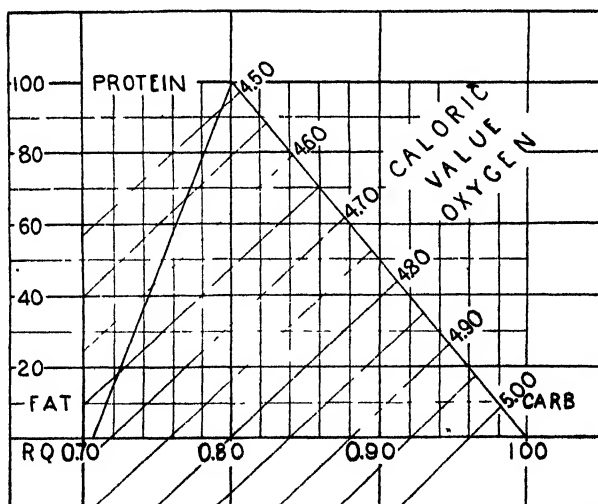


FIG. 2. The calorific value of 1 liter of oxygen in its relationship to the total respiratory quotient (base line) and the percentage of calories from protein (ordinates on the left of chart). The calorific value is found by means of the diagonals which read on the right of the triangle.

carbohydrate percentage changed more than seven points in the third significant figure, and this is well within the experimental error.

There has been a growing tendency among those who use the basal metabolism in clinical medicine to neglect the protein factor and to make all their calculations of the indirect calorimetry from the tables given by Lusk for the calorific value of a liter of oxygen according to the non-protein respiratory quotient. The errors in doing this are shown in Fig. 2, which gives the approxi-

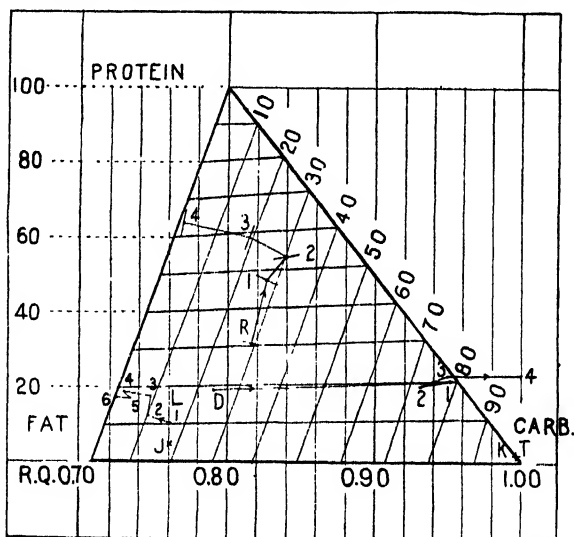


FIG. 3. Triangle used to show changes in metabolism. *R*, the effect of a large protein meal; *D*, the effect of 200 gm. of glucose; *L*, the first 6 days of starvation; *KT* and *J*, low protein diets with muscular exercise.

mate calorific value of a liter of oxygen, plotted according to the respiratory quotient and the percentage of calories furnished by protein. Here the diagonal lines are evenly spaced on account of the nature of the formula. If, for example, the respiratory quotient were 0.90 and protein furnished 20 per cent of the calories, each liter of oxygen used in the metabolism would indicate the production of 4.85 calories.

The triangle shown in Fig. 1 may be used as a "metabolism map." In Fig. 3 we have drawn lines which show on this map the

course of the metabolism during certain experiments in the calorimeter. The starting point *R* gives the position of the basal metabolism of the achondroplastic dwarf, Raphael de P. (4) on March 15, 1916. On this line the point *I* represents his metabolism the next day in the period starting 1 hour after he had finished a breakfast consisting of chopped beef, containing 23.2 gm. of nitrogen. This was an enormous meal for a dwarf weighing 90 pounds. It will be noted that during the 2nd, 3rd, and 4th hours the percentage of calories derived from protein rose until it reached 64 per cent, perhaps the highest percentage ever demonstrated in a respiration experiment on a man. During the 4th hour metabolism was on an exclusively protein-fat basis, similar to that of the Eskimos, but, as we shall see later, safely beyond the zone of ketosis. In this graph we have shown the specific dynamic action and the increase in metabolism due to the protein meal by drawing cross lines whose length is proportional to the height of the metabolism. In this same figure the point *D* represents the position of the usual basal metabolism of the normal control, E. F. D. B. (5). The point *I* on this line shows the position in the calorimeter period starting 1 hour after he had taken 200 gm. of dextrose on May 8, 1914. In the next period there was a slight fall in quotient but a subsequent rise to 1.00. In the lower left-hand corner the line *L* represents the metabolism of Benedict's subject, Levanzin (6), during the first 6 days of his fast.

There are certain positions outside the triangle which are of importance from a metabolic standpoint, as shown in Fig. 4. To the right of the carbohydrate corner there is a zone in which the organism is transforming carbohydrate into fat, and it is of interest to note that this may occur with quotients below 1 when any considerable portion of protein is being metabolized. With very excessive amounts of protein, such as have been attained in dog experiments by Lusk (7), protein can be partially transformed to fat and deposited as such.

To the left of the original triangle is a second triangle which represents the conversion of a portion of the protein molecule into carbohydrate and its storage as glycogen or excretion as glucose. Lusk (8) has shown that the respiratory quotient falls as low as 0.632 if, during the metabolism of 100 gm. of beef protein, 59.41 gm. of glucose, derived from this protein, are excreted in the urine

(dextrose : nitrogen ratio = 3.65). It is only in the lower portion of this triangle that we find patients with diabetes, since they seldom derive more than 35 per cent of their calories from protein.

The line *KA* in the "fat corner" of the original triangle represents the threshold of ketosis, as determined by the equimolecular ratio of fatty acids and glucose, according to Shaffer (9) or the F. A. : G. = 1.5 ratio of Woodyatt (10). This line corresponds to the ingenious and useful graph devised by Hannon and McCann

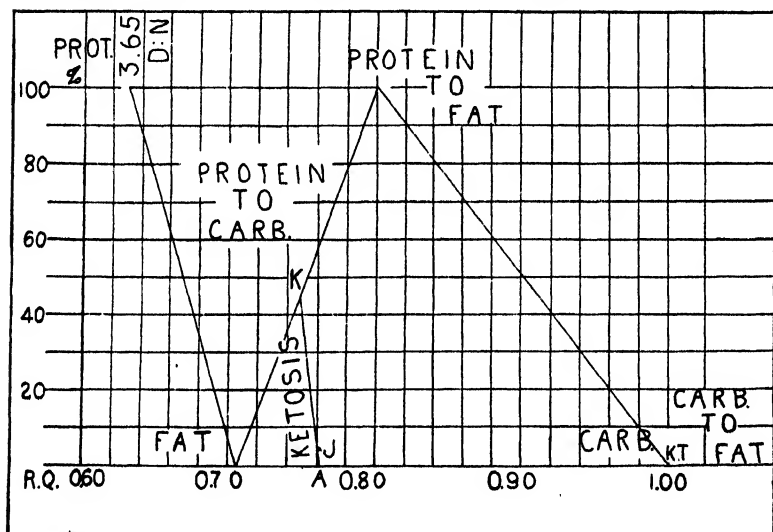


FIG. 4. Zones of metabolism.

(11). To the left of this line diabetic patients, fasting men, or men on a high fat diet, should all theoretically excrete abnormal amounts of ketones.

There is a narrow zone along the bottom of this chart which is probably never reached by man, since we can conceive with difficulty of a state in which protein furnishes less than 1 per cent of the total calories. Perhaps the lowest percentage yet demonstrated is the 1.5 per cent attained by Karl Thomas (12) in a nitrogen minimum experiment in which, on an extremely low protein diet, he performed a large amount of work on an ergometer. This is shown by the point *KT* in Fig. 4. The lowest point near the "fat" end of the scale that I have been able to find was the old

experiment of Atwater and Benedict (13) on the subject, J. C. W., who expended 9,981 calories in a single work day while he was on a diet containing 5,138 calories.

We must remember that this chart shows only the net transformations within the body and expresses the actual loss of protein, fat, and carbohydrate in a given period. It is quite possible that fat is deposited in one part of the body while it is being withdrawn from another organ for purposes of combustion. Krogh and Lindhard (14) have recently emphasized the probability of extensive transformations of this nature.

SUMMARY.

Triangular graphs have been constructed which make it possible to determine rapidly the percentage of calories furnished by protein, fat, and carbohydrates. Using these graphs as maps, one may follow the changes in metabolism caused by disease or by the administration of various diets.

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CLINICAL CALORIMETRY.

THIRTY-SIXTH PAPER.

A GRAPHIC METHOD OF DETERMINING CERTAIN NUMERICAL FACTORS IN METABOLISM.

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As was indicated in the paper immediately preceding, the charts devised by Du Bois¹ may be used to simplify the calculation of the more important numerical values sought in a metabolism determination. The present paper is concerned with the presentation of charts primarily constructed to facilitate such calculation. The use of the charts is first described; the method of constructing them is presented at the end of the paper.

Fig. 1 enables one to determine:

The percentage of the total calories derived (1) from protein, (2) from carbohydrate, and (3) from fat; the percentage of the total oxygen consumed (4) by protein, (5) by carbohydrate, and (6) by fat; provided there are given: (1) the urinary nitrogen (in grams); (2) the total oxygen consumed (in liters); (3) the "total" respiratory quotient (*i.e.* the R. Q. found from the total CO₂ and O₂ without deducting the proportion corresponding to protein).

To determine these factors, the number of grams of urinary nitrogen is to be divided by the number of liters of oxygen. With this quotient as ordinate and the R. Q. as abscissa, there is determined a point, *P*, which commonly falls within the large triangle.²

¹ Du Bois, E. F., *J. Biol. Chem.*, 1924, lix, 43.

² In unusual or pathological states, such as are described in Du Bois' paper, already referred to, the point *P* may fall outside the triangle. In that instance the various factors cannot be determined from these charts.

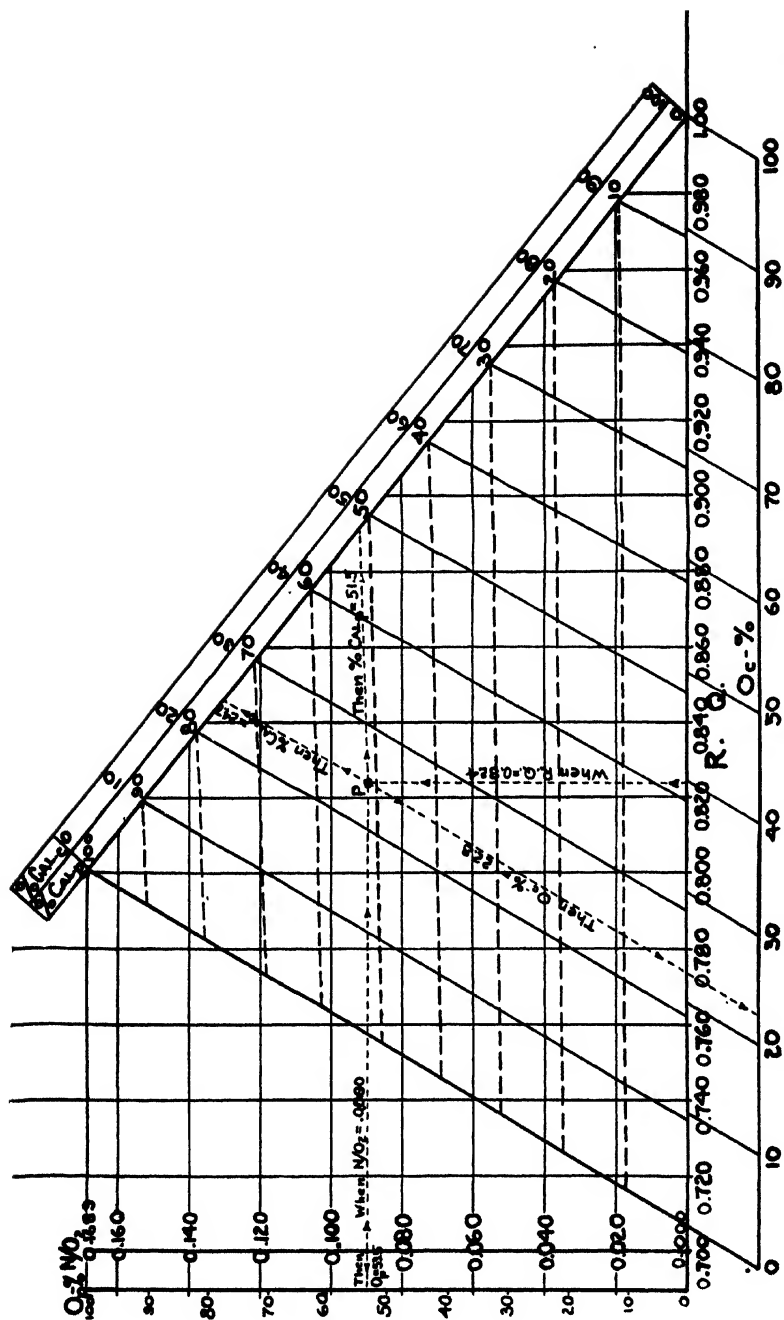


Fig. 1. Chart to give the percentage of calories from protein and carbohydrate and percentage of oxygen used in oxidation of each.

The percentages of the total calories derived from protein and from carbohydrate are both found on the right-hand side of the triangle. For protein a line is drawn through *P*, parallel to the nearest broken line (or approximately parallel to the two nearest). Its intersection with the right-hand scale (using the inner row of figures) gives the percentage of the total calories derived from protein. For carbohydrate the line is drawn through *P*, parallel to the nearest oblique, unbroken line (or approximately parallel to the two nearest) and the position of the point of intersection is read on the outer row of figures.

Subtracting the sum of the protein and carbohydrate percentages from 100 gives the percentage of the total calories derived from fat.

Thus, in the example presented in Fig. 1, where $N:O_2$ has been taken as 0.090 and the R. Q. as 0.824, the values of the percentage calories from protein and from carbohydrate are 51.5 and 24.7, respectively. The percentage of calories from fat = $100 - (51.5 + 24.7) = 23.8$ per cent.

The percentage of the oxygen consumed by each of the three foodstuffs is of less interest than the proportion of the energy which each contributes and it has been subordinated to the latter in the construction of Fig. 1. For protein the percentage of oxygen consumed is read from the vertical scale marked $O_p\%$ opposite any given value of $N:O_2$ on the adjacent scale. To find the percentage of oxygen consumed by carbohydrate, a line is drawn parallel to the left-hand side of the triangle, through the point *P*, referred to above. The intersection of this line with the horizontal scale marked $O_c\%$ gives the desired reading. The percentage of oxygen consumed by fat is given by difference, as before. In the example given in Fig. 1, the percentages of oxygen consumed by protein and by carbohydrate are 53.5 and 22.8 per cent, respectively. Hence the percentage for fat is 23.7 per cent.

Fig. 2 gives the calorific value of a liter of oxygen as a function of the R. Q. and the $N:O_2$ ratio. In using the chart the point *P* is to be found as before, and projected on the scale on the right-hand side of the triangle, by a line parallel to the oblique lines crossing the triangle. The vertical scale, Cal. *p*, gives the *approximate* percentage of calories derived from protein, the maximum error being about 1.3. The scale is not one of equal parts. In

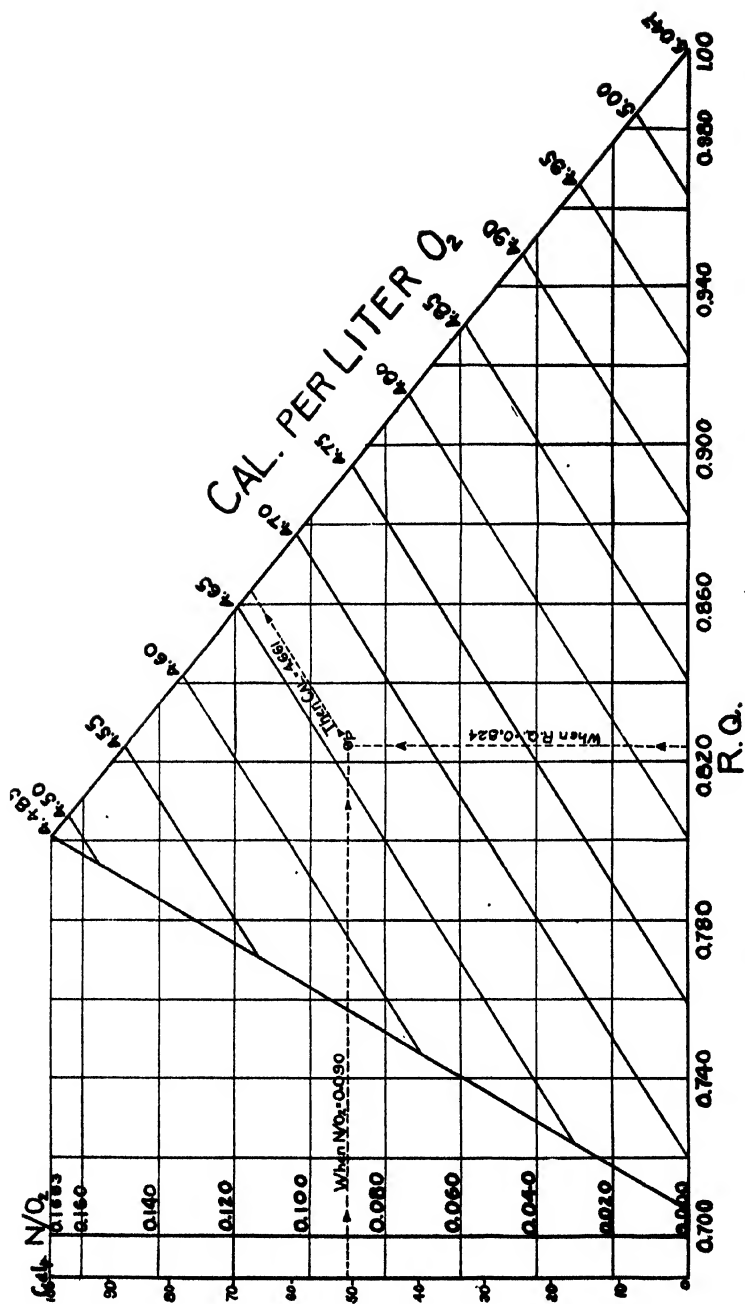


FIG. 2. Chart showing the caloric value of a liter of oxygen according to the total R. Q. and the relationship of urinary nitrogen and total oxygen consumption.

the example given in Fig. 2, where, conveniently, the same values of $N:O_2$ and R. Q. have been taken, as were used before, the calorific value of a liter of oxygen is found to be 4.661 (cals.).

Charts could be constructed to give the amount (grams) of foodstuff metabolized per liter of oxygen, and the percentage of protein, carbohydrate, and fat.

Construction of the Charts.

The coordinate axes in Figs. 1 and 2 are identical and the triangles are homologous, so that the two graphs could be superimposed one upon the other but for the confusing multiplicity of scales and projection lines which would result. More simply, the $O_p\%$ and the $N:O_2$ scales may be combined as a single line with a row of figures on either side; so, likewise, the $O_c\%$ and the R. Q. scales. On the other hand, Fig. 1 may be resolved into three separate charts, one for the percentage of calories from protein, another for the percentage of calories from carbohydrate, and a third for the percentage of oxygen consumed by carbohydrate. Where, as in Fig. 2, there is a set of parallel projection lines, they may be dispensed with, and the point P , projected on the proper scale by the edge of a T-square which can be moved along the edge of a drawing board to which the chart is fastened at the proper angle. On such a chart, a single line of the parallel set (here, conveniently the one from the lower left-hand corner to the point, 4.686, on the right-hand scale) may be drawn for reference.

The use of different colors for the projection lines and numerals will also simplify the use of the charts.

The charts as here presented show too few scale divisions and projection lines to permit accurate reading to three, or even to two, significant figures. The space may, however, be subdivided by interpolation. This has here been omitted in order to simplify the appearance of the charts and to render their exposition easier.

As was done with the original tracing, the charts may be advantageously constructed on a much larger scale than space here will permit.

The construction of the charts is possible in a variety of ways, some of which have been indicated above; only the method presented in this paper is detailed below.

The symbols and constants employed in the formulas used in the construction of the charts are:

Legend of Symbols.

- N = grams of urinary nitrogen for the given period.
 O_2 = liters of oxygen consumed during the same period.
 R. Q. = the respiratory quotient for the period.
 P, C, and F = the percentages of the calories derived from protein, from carbohydrate, and from fat, respectively.
 p, c, and f = the percentages of oxygen consumed by each.
 ρ , γ , and ϕ = the percentages of each in the metabolic mixture.

Constants.

Liters of oxygen per gram of urinary nitrogen, metabolized when protein alone is burning = 5.94.

R. Q. for fat, protein, and carbohydrate = 0.707, 0.801, and 1.000, respectively.

Calorific values of a liter of oxygen for protein, fat, and carbohydrate = 4.485, 4.686, and 5.047, respectively.

Grams of substance burned per liter of oxygen, for fat, protein, and carbohydrate = 0.50, 1.03, and 1.21, respectively.

Due to variations in composition of protein, carbohydrate, and fat, the "constants" given are accurate to only two significant figures, with the exception of the calorific value of a liter of oxygen, which is accurate to three, and the R. Q. for carbohydrate, which is theoretically exact. Nevertheless, with the exception of the values for grams of substance per liter of oxygen, the extra figure is retained, the values given being the commonly accepted ones.³

Construction of Fig. 1.—Using a convenient scale, the R. Q. is plotted along the axis of abscissæ, and the values of the quotient, N:O₂, along the axis of ordinates. The O_p-% scale is constructed by drawing a line parallel to the N:O₂ scale and dividing the distance between 0 and 0.1683 into 100 equal parts. A centimeter scale or a strip of decimal cross-section paper will prove useful in performing this construction.

The coordinates of the vertices of the large triangle are (0.707, 0), (0.801, 0.1683), and (1.000, 0). These points are joined by straight lines.

³ Lusk, G. The elements of the science of nutrition, Philadelphia, 3rd edition, 1917.

The $O_c\%$ scale is constructed on a line parallel to the R. Q. scale. The zero point on the scale is found by continuing the left-hand side of the triangle until it intersects the scale. The scale is the length of the base of the triangle, and is divided into 100 equal parts.

To find points on the sides of the triangle, the following formulas are used. Formulas are given which determine both coordinates, and since the points must lie on one or the other side of the triangle, one formula serves as a check on the other, only one coordinate being required to determine the point.

To construct the scale on the right-hand side of the triangle: This scale reads P and C , and along this line $P + C = 100$. The coordinates of any given point, $P = 100 - C$, are:

$$(1) \quad N:O_2 = \frac{0.8497 P}{0.562 P + 448.5} \qquad (2) \quad R. Q. = \frac{404.3 + 0.442 C}{504.7 - 0.582 C}$$

The coordinates, $N:O_2$ and R. Q., of that point on the left-hand side of the triangle to which a line is drawn from the point, $P = 100 - C$, on the right-hand scale, are:

$$(3) \quad N:O_2 = \frac{0.7889 P}{0.201 P + 448.5} \qquad (4) \quad R. Q. = \frac{375.3 - 0.582 C}{468.6 - 0.201 C}$$

The point on the base of the triangle to which a line is drawn from the point, $C = 100 - P$, on the right-hand scale, is given by:

$$(5) \quad R. Q. = \frac{468.6 - 1.118 P}{468.6 + 0.361 P} \quad \text{or} \quad (6) \quad c = \frac{468.6 C}{504.7 - 0.361 C}$$

These formulas cannot be used to calculate the values of P and C corresponding to a point within the triangle. They are simply used for constructing the chart. It must be remembered that, for a point within the triangle, $P + C + F = 100$, and $p + c + f = 100$, and that there would be three variables in every formula.

It is to be noted that the scale on the right-hand side of the triangle is *not one of equal parts*, and that the projection lines are *not parallel*. Were the scale constructed of equal parts, and the projection lines drawn parallel, the error in the percentage would amount to a maximum of about 3.0. With charts reading percentage mass instead of calories, the error due to such incorrect construction would be much greater.

Construction of Fig. 2.—The triangle, and the N:O₂ and R.Q. scales, are constructed as before. The Cal. p scale is constructed empirically, using average values from Fig. 1. The right-hand scale is one of equal parts, from 4.485 at the apex to 5.047 at the base. The projection lines are all parallel, the easiest to construct being the one from the lower left-hand corner of the triangle to the point, 4.686, on the right-hand scale.

Charts similar to Figs. 1 and 2 may be constructed to give percentage, and total, mass instead of calories. The triangle, and the N:O₂ and R.Q. scales, would be identical with those used in Figs. 1 and 2. For the chart giving percentages of protein and carbohydrate, the formulas corresponding to those used for Fig. 1 are:

$$(1a) \quad \text{N:O}_2 = \frac{0.204 \rho}{103 + 0.18 \rho} \qquad (2a) \quad \text{R. Q.} = \frac{97 + 0.06 \gamma}{121 - 0.18 \gamma}$$

$$(3a) \quad \text{N:O}_2 = \frac{0.084 \rho}{103 - 0.53 \rho} \qquad (4a) \quad \text{R. Q.} = \frac{40 + 0.33 \gamma}{50 + 0.53 \gamma}$$

$$(5a) \quad \text{R. Q.} = \frac{50 + 0.36 \rho}{50 + 0.71 \rho} \quad \text{or} \quad (6a) \quad c = \frac{50 \gamma}{121 - 0.71 \gamma}$$

The chart corresponding to Fig. 2, which gives the grams of foodstuff per liter of oxygen, would have the scale of grams per liter (equal parts) along the base of the triangle, from 0.50 at the left-end, to 1.21 at the right. The apex is connected with the point 1.03 on the base, and the other lines drawn parallel to this one.

To present the derivation of the above formulas and proof of the validity of the construction would be beyond the scope and purpose of this paper.

THE METABOLISM OF SULFUR.

VI. THE OXIDATION OF CYSTINE IN THE ANIMAL ORGANISM.

SECOND PAPER.

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(Received for publication, December 3, 1923.)

In a former paper (1) it has been suggested that oxidation of the sulfur of the cystine molecule by the animal organism is closely related to the process of deamination of the amino acid or the oxidation of the deamination products. The amino group of cystine was "protected" by conjugation with phenylisocyanate and the behavior of the resulting phenyluraminocystine was studied. When this was administered either orally or subcutaneously to rabbits, the sulfur of the complex was not oxidized normally but was recovered, for the most part, in the urine as unoxidized sulfur. Cystine under similar conditions was completely oxidized and the sulfur was eliminated by the kidneys as sulfate sulfur. In a subsequent study (2) we were able to demonstrate that a portion of the administered phenyluraminocystine appeared in the urine in changed form, having been reduced to phenyluraminocysteine. It was suggested that the first step in the normal catabolism of cystine is a reduction to cysteine and that oxidation having been prevented by "protection" of the amino group, the reduced phenyluraminocysteine was eliminated by the kidneys. The importance of the reversible reaction $\text{cystine} \rightleftharpoons \text{cysteine}$ as a factor in oxidation in the body has been emphasized by Hopkins and his coworkers (3) and by others (4).

Recently Shiple, Rose, and Sherwin (5) have reported similar studies on the oxidation by the animal organism of derivatives of cystine with the amino and carboxyl groups protected, phenylacetyl cystine, phenyluraminocystine, and its hydantoin.

Full details of this work are not available. They report that conjugation of the amino group did not completely prevent oxidation of the sulfur. It should be noted, however, that as far as stated in their preliminary report, the compounds were administered orally. We have noted previously (1) that, after *oral* administration of phenyluraminocystine, a limited oxidation of the sulfur fraction of the molecule occurred, while, after subcutaneous injection, there was no apparent evidence of oxidation. This slight degree of oxidation was considered to be the result directly or indirectly of bacterial action in the intestine.

In order to obtain further evidence concerning the factors which are involved in the oxidation of the sulfur fraction of the cystine molecule, we have studied the behavior of another derivative of cystine, dibenzoylcystine, in which the amino group is protected from deamination by conjugation with the benzoyl group. It has been shown by Magnus-Levy (6) that benzoylated amino acids are eliminated without cleavage of the benzoyl group and deamination. Quantitative data as to the extent of the conversion of cystine to cysteine by the organism in the case of phenyluraminocystine and dibenzoylcystine have also been obtained.

EXPERIMENTAL.

The dibenzoylcystine was prepared by the benzoylation of cystine in the usual manner (7). The product was recrystallized from alcohol to which a sufficient amount of water had been added to start crystallization. Because of the marked tendency of dibenzoylcystine to form a gel with water (8) some difficulty was experienced in the crystallization from water and alcohol. The purity of the product obtained was established by analysis for sulfur.

The experimental animals were rabbits which were maintained on a constant diet of cabbage and oats or milk and oats. The urine was collected in 24 hour periods as usual. Total nitrogen was determined by the Kjeldahl method, total sulfate sulfur by the method of Folin, and total sulfur by the Denis modification of the Benedict method. Unoxidized sulfur was obtained by difference. The dibenzoylcystine was administered as the sodium salt, prepared by dissolving the free acid in sodium carbonate or hydroxide.

In the latter experiments (Tables IV to VI) in which an attempt was made to determine the extent to which the cystine derivative administered had been converted into a cysteine derivative in the organism, the method of Looney (9) for the determination of cystine in urine was used.

This method depends upon the fact that, while cystine alone does not react with phosphotungstic acid (the uric acid reagent of Folin and Denis), in the presence of sodium sulfite it is reduced to cysteine which gives a deep blue color with the reagent. Uric acid, polyphenols, and possibly other constituents of the urine will react with the reagent to give a deep blue color, but their reaction is not influenced by the presence of sulfite. The cystine is calculated from the increased color after the addition of the sulfite. In our experiments we have determined the amounts of those substances in the normal urines which react with the uric acid reagent without the addition of sulfite and have expressed these as "free mercapto" groups calculated in terms of the standard, cystine. On the experimental days, when cystine derivatives were fed, marked increase in the "free mercapto" groups was noted and since the cystine derivatives fed should not influence the excretion of substances normally present and giving this reaction, we have considered the increased color to be due to the excretion of cysteine derivatives and have accordingly calculated the extra "free mercapto" groups present. Similarly, we have calculated the "total mercapto" groups present by measuring the color produced after the addition of sulfite to the urine. By difference we have obtained the "combined mercapto" groups present (*i.e.* "mercapto" groups liberated by the reducing agent) and have regarded the increases observed in this case as due to the presence of the unchanged cystine derivatives administered in the urine. For convenience in comparison, all results are calculated in terms of cystine.

The main limitation of this method was found to be the rather rapid fading of the blue color when no sulfite was present. For this reason the "free mercapto" group figures must be regarded as minimal. Control experiments with the phenyluraminocystine alone and with phenyluraminocystine added to urine, demonstrated that the method was adequate for approximate results, although the rate of reduction of the phenyluramino derivative

of cystine appeared to be slightly less rapid than that of cystine itself. Hydrogen sulfide or simple aliphatic mercaptans, if present, would also react with the reagent and appear as "free mercapto" groups in our quantitative data. However, neither in these nor in our previous experiments (1, 2) have we been able to detect the presence of compounds of this type.

DISCUSSION.

The partition of sulfur following the administration of dibenzoylcystine to rabbits is shown in Tables I to III. The results are in general similar to those previously obtained when phenyluraminocystine was administered (1). A certain amount of oxidation of the sulfur was observed after feeding the dibenzoylcystine, but no oxidation occurred when the compound was injected subcutaneously. Thus with Rabbit 970 (Table I) after feeding an amount of dibenzoylcystine equivalent to 0.133 gm. of sulfur, approximately 60 per cent of the sulfur administered was recovered in the urine as extra total sulfur. Of this amount of extra sulfur, 62.5 per cent was accounted for in the increase in total sulfate sulfur and 37.5 per cent in the unoxidized sulfur. Similarly, in a second experiment with the same animal (March 25, Table I), in which the benzoyl derivative was fed, the total recovery of extra sulfur corresponded to 64 per cent of the sulfur fed, which was distributed between sulfate sulfur and unoxidized sulfur about as in the preceding experiment (61.1 and 38.9 per cent, respectively). When, however, benzoylcystine was injected, although the amount of the injected sulfur which was recovered in the urine was approximately the same as when administered *per os*, the greater part of the extra sulfur (78.7 per cent) appeared as unoxidized sulfur, and a negligible increase (8 mg.) of sulfate sulfur was noted. Such a small variation in sulfate sulfur is no greater than that frequently observed on normal days and has little significance.

With Rabbit 971 (Table II) similar results were obtained. After subcutaneous and oral administrations of the same amounts of dibenzoylcystine, 60.8 and 56.3 per cent of the sulfur ingested were recovered in the urine. Of this extra sulfur, 93 per cent appeared as unoxidized sulfur, when the benzoyl derivative was injected, while 56 and 44 per cent were present as sulfate and unoxidized sulfur, respectively, after feeding. The results with

TABLE I.

Rabbit 970. Blue male. Weight 2.4 kilos. Diet: 100 cc. of milk and 30 gm. of oats daily.

Date.	Total S.	Total SO ₄ S.	Unoxidised S.	Remarks.
<i>1922</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	
Feb. 19	0.085	0.068	0.017	
" 20	0.065	0.050	0.015	
" 21	0.067	0.047	0.020	
" 22	0.070	0.051	0.019	
" 23	0.144	0.098	0.046	{0.933 gm. dibenzoylcystine as Na salt <i>per os</i> . (S = 0.133 gm.)
" 24	0.073	0.059	0.014	
" 25	0.068	0.055	0.013	
" 26	0.055	0.039	0.016	
" 27	0.057	0.043	0.014	
" 28	0.064	0.050	0.014	{0.933 gm. dibenzoylcystine as Na salt subcutaneously. (S = 0.133 gm.)
Mar. 1	0.143	0.066	0.087	
" 2	0.067	0.052	0.015	
" 3	0.071	0.056	0.015	
" 4	0.085	0.068	0.017	{0.5 gm. cystine as Na salt subcutaneously. (S = 0.133 gm.)
" 5	0.087	0.065	0.022	
" 6	0.085	0.066	0.019	
" 7	0.085	0.066	0.019	
" 13	0.079	0.065	0.014	
" 14	0.068	0.055	0.013	
" 15	0.152	0.127	0.025	{0.5 gm. cystine as Na salt subcutaneously. (S = 0.133 gm.)
" 16	0.084	0.068	0.016	
" 17	0.071	0.058	0.013	
" 18	0.068	0.055	0.013	
" 22	0.077	0.061	0.016	
" 23	0.057	0.046	0.011	
" 24	0.063	0.051	0.012	
" 25	0.149	0.100	0.049	{0.933 gm. dibenzoylcystine as Na salt <i>per os</i> . (S = 0.133 gm.)
" 26	0.059	0.047	0.012	
" 27	0.065	0.053	0.012	
" 28	0.069	0.056	0.013	

Rabbit F (Table III) were similar. In this experiment, there was some irregularity in the normal sulfur excretion which makes it somewhat difficult to calculate the percentage of recovery and of oxidation of the sulfur.

It seems probable that the differences in the oxidation of the sulfur after administration of the dibenzoylcystine enterally and

parenterally may be explained by cleavage of the benzoyl group from the molecule in the intestine by enzymes of the tract or by bacterial

TABLE II.

Rabbit 971. Brown female. Weight 2.7 kilos. Diet: 100 cc. of milk and 30 gm. of oats daily.

Date.	Total S.	Total SO ₄ S.	Unoxidised S.	Remarks.
<i>1922</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	
Apr. 1	0.076	0.060	0.016	
" 2	0.068	0.053	0.015	
" 3	0.067	0.052	0.015	
" 4	0.101	0.077	0.024	{ 0.5 gm. cystine as hydrochloride subcutaneously. (S = 0.133 gm.)
" 5	0.065	0.051	0.014	
" 6	0.068	0.051	0.017	
" 7	0.067	0.050	0.017	
" 8	0.053	0.039	0.014	
" 9	0.074	0.057	0.017	{ 0.933 gm. dibenzoylcystine as Na salt subcutaneously. (S = 0.133 gm.)
" 10	0.146	0.064	0.092	
" 11	0.054	0.039	0.015	
" 12	0.064	0.048	0.016	
" 13	0.065	0.050	0.015	{ 0.933 gm. dibenzoylcystine in suspension in water <i>per os</i> . (S = 0.133 gm.)
" 14	0.140	0.091	0.049	
" 15	0.061	0.045	0.016	
" 16	0.065	0.047	0.018	
" 17	0.064	0.047	0.017	

TABLE III.

Rabbit F. Gray female. Weight 1.7 kilos. Diet: 150 cc. of milk and 30 gm. of oats daily.

Date.	Total S.	Total SO ₄ S.	Unoxidised S.	Remarks.
<i>1922</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	
Mar. 21	0.091	0.069	0.022	
" 22	0.090	0.068	0.022	
" 23	0.053	0.039	0.014	
" 24	0.123	0.082	0.041	{ 0.89 gm. dibenzoylcystine as Na salt <i>per os</i> . (S = 0.129 gm.)
" 25	0.130	0.094	0.036	
" 26	0.047	0.036	0.011	
" 27	0.055	0.042	0.013	

enzymes. Magnus-Levy (6) *injected* the benzoyl derivatives of a number of amino acids and found that, with one exception, they were excreted unchanged by the kidneys. Hippuric acid was

excreted after the injection of a benzoyl derivative of an unidentified amino acid from the leucine fraction of a protein hydrolysate.¹ Epstein and Bookman (10) administered benzoyl leucine to rabbits. Since they fail to state, as far as we have been able to discover, the method of administration, it is difficult to interpret their data. In a later paper (11), benzoylalanine and benzoyl glucose were *fed* to rabbits. The hippuric acid output was greater than on normal days, but the increases were not so large as to necessitate a complete cleavage of the benzoyl group to benzoic acid and conjugation in order to account for them. In one experiment,² the rise in hippuric acid excretion is such as to be explained by a cleavage of about 50 per cent of the benzoyl group. The method used for the determination of the hippuric acid is not stated in either of their papers cited. It is to be assumed that the method used involved isolation of the hippuric acid, since other methods (*e.g.* that of Folin and Flanders) would determine the total benzoic acid and would convert benzoyl derivatives into benzoic acid in the analytical procedure. We believe, in view of the fact that Magnus-Levy observed no hippuric acid formation and no hydrolysis of the benzoylated amino acids after *injection*, that the results of Epstein and Bookman indicate a partial cleavage of the benzoyl derivative in the alimentary canal.³ Our own experiments with benzoylcystine would support such a conclusion.

The results of the administration of dibenzoylcystine to rabbits are in confirmation of our earlier work (1) and that of Sherwin and collaborators (5) and indicate that oxidation of the cystine sulfur is not readily effected when the amino group of the molecule is protected from deamination.

¹ In an earlier paper (Magnus-Levy, A., *Munch. med. Woch.*, 1905, lii, 2168), Magnus-Levy stated that this result was obtained with benzoyl leucine. Subsequently he pointed out (6) that this was erroneous and that the substance considered to be benzoyl leucine was the benzoyl derivative of an unidentified constituent of the leucine fraction. Benzoyl leucine was excreted unchanged. Epstein and Bookman (10, 11) cite only the earlier paper and were evidently not aware of the later correction.

² Epstein and Bookman (11), Table I, p. 457.

³ Further studies of the behavior of benzoyl derivatives of amino acids are in progress in this laboratory.

In a former paper (2) we reported the occurrence of phenyluraminocysteine in the urine of rabbits after the administration of phenyluraminocysteine. We were not able to obtain the cysteine derivative itself in pure form, but reached this conclusion by analysis of the copper salt and by other means. Recently, Shipley and Sherwin (12) have synthesized phenyluraminocysteine. The

TABLE IV.

Rabbit B. White male. Daily diet: 65 gm. of oats and 100 gm. of cabbage.

Date	Weight.	"Mercapto" group calculated as cystine.			Extra "mercapto" groups as cystine.	
		Free.	Total.	Combined	Free.	Combined.
1943	kg.	mg.	mg.	mg.	mg.	mg.
Oct. 19	2.70	43.1	73.9	30.8		
" 20	2.65	48.9	67.7	18.8		
" 21	2.75	45.0	62.3	17.3		
" 22*	2.70	185.1	340.0	154.9	140.6	133.7
" 23	2.70	85.7	175.7	90.0	41.2	68.9
" 24	2.65	58.1	103.6	45.5	13.6	24.4
" 25	2.65	44.8	70.2	25.4		
" 26	2.60	43.2	65.6	22.4		
" 27†	2.60	43.7	76.9	33.2	0.0	12.1
" 28	2.55	38.2	59.1	20.9		
" 29‡	2.55	91.7	187.8	96.1	57.2	75.0
" 30	2.55	43.4	65.1	21.7		
" 31	2.55	42.8	60.0	17.2		
Nov. 1§	2.55	296.9	571.4	274.5	252.4	253.4
" 2	2.50	46.2	66.7	20.5	1.7	0.0
" 3	2.50	42.9	63.2	20.3		
" 4	2.45	44.8	64.5	19.7		

* 1.0 gm. of phenyluraminocysteine as Na salt *per os* (\approx 0.5 gm. of cystine).

† 0.5 gm. of cystine as Na salt *per os*.

‡ 0.933 gm. of dibenzoylcysteine as Na salt *per os* (\approx 0.5 gm. of cystine).

§ 1.0 gm. of phenyluraminocysteine as Na salt subcutaneously (\approx 0.5 gm. of cystine).

properties of the pure substance corresponded very closely to those of the substance which we obtained, but which we were unable to isolate in pure condition from the urine.

A quantitative study of the elimination of cysteine derivatives following the administration of phenyluraminocysteine and di-

benzoylcystine is reported in Tables IV to VI. The quantitative data, which were obtained by the method of Looney as previously

TABLE V.

Rabbit C. Brown female. Daily diet: 65 gm. of oats and 100 gm. of cabbage.

Date.	Weight.	"Mercapto" group calculated as cystine.			Extra "mercapto" groups as cystine.	
		Free.	Total.	Combined.	Free.	Combined.
1925	kg.	mg.	mg.	mg.	mg.	mg.
Nov. 1	3.2	40.1	82.8	42.7		
" 2	3.25	50.1	75.6	25.5		
" 3	3.25	62.5	91.7	29.2		
" 4	3.25	64.9	85.6	20.7		
" 5*	3.15	170.7	276.9	106.2	115.0	83.2
" 6	3.00	99.5	147.8	48.3	43.8	25.3
" 7	2.90	96.1	142.6	46.5	40.4	23.5
" 8	2.85	50.7	71.4	20.7		
" 9	2.90	47.2	66.2	19.0		
" 10†	2.90	363.1	696.5	333.4	307.4	310.4
" 11	2.95	52.5	78.0	25.5		
" 12	3.00	62.4	82.0	19.6		

* 1.0 gm. of phenyluraminocystine as Na salt *per os* (\approx 0.5 gm. of cystine).

† 1.0 gm. of phenyluraminocystine as Na salt subcutaneously.

TABLE VI.

Rabbit B. White male. Daily diet: 65 gm. of oats and 100 gm. of cabbage.

Date.	Weight.	"Mercapto" group calculated as cystine.			Extra "mercapto" groups as cystine.	
		Free.	Total.	Combined.	Free.	Combined.
1925	kg.	mg.	mg.	mg.	mg.	mg.
Nov. 9	2.50	39.0	70.8	31.8		
" 10	2.55	32.3	80.0	47.7		
" 11	2.55	36.3	52.5	16.2		
" 12*	2.55	102.1	242.6	140.5	65.3	112.0
" 13	2.55	40.0	58.5	18.5		

* 0.933 gm. of dibenzoylcystine as Na salt *per os* (partly in suspension) (\approx 0.5 gm. of cystine).

discussed in this paper, were checked by qualitative tests for mercapto groups. The nitroprusside-ammonia reaction was

carried out on all experimental urines and the findings in all cases corresponded to the quantitative data. In addition, after the administration of phenyluraminocystine, the elimination of a cysteine derivative was demonstrated by the reaction with copper sulfate previously described (2).

In the experiments recorded in Table IV, on the day of the oral administration of 1.0 gm. of phenyluraminocystine, approximately 55 per cent of the cystine derivative fed appeared in the urine as extra total mercapto groups. Of this, 51 per cent was present as a cysteine derivative and 49 per cent as a cystine derivative. There was a lag in excretion extending over 2 days during which time both cystine and cysteine derivatives were excreted. A similar lag in excretion was noted in our earlier work (1). When cystine itself was fed, no increase in the free mercapto groups was noted and only a slight rise in combined mercapto groups, corresponding to cystine. This is in harmony with the accepted belief that cystine in moderate amounts is completely oxidized in the organism. When phenyluraminocystine was injected, the data showed an elimination of the substance which was complete, both as free and combined mercapto groups. In fact, the total mercapto groups indicated a recovery of slightly more than 100 per cent. As in the feeding experiments, the distribution between free and combined mercapto groups was nearly equal. Similar results were obtained for phenyluraminocystine with Rabbit C (Table V). The recovery of the cystine derivative fed as extra mercapto groups (free and combined) on the day of feeding and on the 2 following days was approximately 70 per cent of that fed. This figure is comparable with and checks well with the amount of sulfur recovered as unoxidized sulfur in previous experiments (1). Of these extra total mercapto groups, 40 per cent was eliminated on the day of feeding. On this day 58 per cent of the extra mercapto groups was in the form of a cysteine derivative (free mercapto) and 42 per cent as a cystine derivative (combined mercapto). After subcutaneous injection, the distribution of free and combined mercapto groups was equal and, as in the previous experiment, somewhat more than the amount fed was recovered. As pointed out earlier, we do not consider the method absolutely quantitative, but capable of giving approximate data only.

The data with dibenzoylcystine (Tables IV and VI) include feeding experiments only. They are in accord with the conclusions already reached from a consideration of the sulfur partition in the urine after dibenzoylcystine feeding in that they show a lower percentage of the sulfur fed recovered in the urine as unoxidized sulfur than when phenyluraminocystine was fed. Thus with Rabbit B (Table IV) only 26 per cent of the sulfur fed appeared as total mercapto groups and of this, 43 per cent was eliminated as free mercapto or cysteine sulfur. In a second experiment (Table VI) the total recovery was slightly higher, about 35 per cent, and the percentage excreted as free mercapto, 36.7, was slightly lower. The data obtained with both compounds afford more accurate quantitative evidence as to the extent of the conversion of cystine to cysteine than has been available heretofore.

Further results which concerned the velocity of the transformation of cystine to cysteine and its elimination were also obtained. A rabbit received 0.55 gm. of phenyluraminocystine (=0.275 gm. of cystine) as the sodium salt *per os* and the urine excreted in the next 2 hours was collected and analyzed. The urine gave a strong nitroprusside-ammonia test and a heavy gray-black precipitate with copper sulfate. On analysis, 162.2 mg. of total mercapto groups (calculated as cystine) were found which were equivalent to a recovery of about 59 per cent. of the cystine fed. Of this 84.9 mg. (52.3 per cent) were present as free mercapto groups (cysteine) and 77.3 mg. (47.7 per cent) as combined mercapto groups (cystine). It was thought possible that the conversion of cystine to cysteine observed previously in 24 hour samples might be due to reduction of the cystine after the urine was secreted, either in the urinary bladder or after voiding, in the cage. The figures, just cited, however, which indicate a very rapid conversion to cysteine, would speak against such a possibility. As further evidence on this point, this same urine was allowed to stand 20 hours at 40° in an incubator without any preservative and again analyzed. There was a slight diminution of the total mercapto groups (144.6 mg.) and a *slight rise in combined mercapto groups* (90.3 mg.), indicating that a small amount of oxidation of the cysteine derivative had occurred during the period of incubation. From these and other similar experiments, we are led to believe that the conversion of the cystine derivative

to the cysteine derivative had been effected before the excretion of the compound by the kidneys.

In the experiment just described, the animal was slaughtered after the urine was collected and the blood was examined for the presence of both cystine and cysteine derivatives. The results were negative. This, however, was not unexpected, in view of the large excretion of the sulfur compounds noted in the urine secreted during the 2 hour period which had elapsed between the ingestion of the phenyluraminocystine and the death of the animal.

SUMMARY.

1. After the subcutaneous injection of dibenzoylcystine into rabbits, no oxidation of the sulfur of the cystine molecule was observed, but the "extra" sulfur eliminated was almost wholly in the unoxidized sulfur fraction of the urine. This is in harmony with the theory previously suggested by us (1) that the oxidation of the sulfur cannot take place normally if deamination be prevented.

2. Some oxidation of the sulfur of the cystine molecule occurred after feeding dibenzoylcystine to rabbits and the increase in the "extra" sulfur eliminated was divided between the total sulfate sulfur and unoxidized sulfur fractions of the urine. It is considered probable that some of the benzoyl groups were split off in the alimentary canal and that oxidation of the cystine liberated proceeded normally.

3. Quantitative data concerning the extent of the conversion of cystine to cysteine after the administration of phenyluraminocystine to rabbits are presented. About 50 per cent of that portion of the compound which was recovered in the urine was converted to a cysteine derivative and an equal quantity excreted unchanged (as a cystine derivative). Similar results were obtained from a study of the transformation of dibenzoylcystine to a cysteine derivative in its passage through the animal body.

4. It is believed that this conversion of cystine to cysteine occurred in the organism and not in the urine after its secretion.

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ON THE USE OF FORMALDEHYDE FOR THE PRESERVATION OF BLOOD SPECIMENS.

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(Received for publication, November 21, 1923.)

The use of formaldehyde as a preservative for bloods intended for blood sugar determination by the revised Folin-Wu¹ method was recommended by Denis and Aldrich.² These authors use commercial "formalin," containing approximately 40 per cent of formaldehyde, their procedure calling for the addition of 1 drop of the reagent to 5 ml. of blood. Denis and Aldrich claim that formaldehyde will not influence the alkaline copper tartrate solution used in the Folin-Wu procedure, even if added in amounts ten times greater than the quantity originally mentioned. They also state

" . . . that if to 2 cc. of the tungstic acid filtrate there is added 1 drop of formaldehyde, and the sugar determination is made on this portion of filtrate in the usual way, results will be secured which are identical with those obtained in portions of the same filtrate to which no formaldehyde has been added."

It has been found in the present work that many samples of formaldehyde will materially influence the results if used in any of the quantities mentioned by Denis and Aldrich, and the following experiments are offered in this connection.

Six samples of formaldehyde were obtained from various sources, and were numbered from one to six successively. Nos. 1, 2, and 3 are formaldehydes of U. S. P. standard, No. 4 is marked c. p. and had been opened about a year ago, Nos. 5 and 6 are fresh specimens, Merck's White Label and Blue Label, respec-

¹ Folin, O., and Wu, H., *J. Biol. Chem.*, 1920, xli, 367.

² Denis, W., and Aldrich, M., *J. Biol. Chem.*, 1920, xliv, 203.

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TABLE I.

Experimental Results Showing the Effect of Adding Formaldehyde to Glucose Solution, Tungstic Acid Filtrate, and Blood.

Material used.	Formaldehyde sample No.	Amount of formaldehyde.	Amount of reduction expressed in mg. glucose.
Glucose solution.		<i>drops</i>	<i>mg.</i>
		None.	0.080 Theory (0.080)
	1	6 in 20 ml.	0.133
	2	6 " 20 "	0.139
	3	6 " 20 "	0.117
	4	6 " 20 "	0.088
	5	6 " 20 "	0.126
	6	6 " 20 "	0.149
Glucose solution.		None.	0.400 Theory (0.400)
	1	1 in 2 ml.	0.470
	2	1 " 2 "	0.437
	3	1 " 2 "	0.526
	4	1 " 2 "	0.394
	5	1 " 2 "	0.500
	6	1 " 2 "	0.512
Tungstic acid filtrate.		None.	0.200 Theory (0.200)
	1	1 in 2 ml.	0.288
	2	1 " 2 "	0.303
	3	1 " 2 "	0.292
	4	1 " 2 "	0.208
	5	1 " 2 "	0.370
	6	1 " 2 "	0.309
Blood.			Per 100 ml. blood.
			<i>mg.</i>
		None.	78
	1	1 in 5 ml.	143
	1	4 " 5 "	216
Blood.	5	4 " 5 "	200
		None.	62
	1	1 in 2 ml.	107
	2	1 " 2 "	91
	3	1 " 2 "	94
	4	1 " 2 "	69
	5	1 " 2 "	106
	6	1 " 2 "	89
Blood.		None.	532
	3	1 in 5 ml.	544
	3	3 " 5 "	556

TABLE I—*Concluded.*

Material used.	Formaldehyde sample No.	Amount of formaldehyde.	Amount of reduction per 100 ml. blood.
		<i>drops</i>	<i>mg.</i>
Blood.		None.	278
	4	1 in 2 ml.	280
	4	5 " 2 "	280
Blood.		None.	38
	2	1 in 5 ml.	43
	2	4 " 5 "	70
Blood.		None.	104
	6	1 in 5 ml.	130

tively. All samples are of approximately the same specific gravity and usual percentage.

The drops were added from a standard pipette filled to a certain mark on the lower stem. The delivery was 27 drops to 1 gm. The same rate of delivery was maintained in all experiments. The revised method of Folin-Wu¹ was used throughout.

The results presented in Table I show the influence of formaldehyde on various materials. We first studied the effect of adding divers amounts of the different formaldehydes to pure glucose solutions. It will be noticed that the increase, expressed in terms of glucose, is more than 40 per cent in several cases. Formaldehyde sample No. 4 caused no appreciable difference.

Next, the influence of formaldehyde on tungstic acid filtrates was investigated. Blood was allowed to stand in the ice box until no more sugar was detected by the Folin-Wu method. The bulk of the blood was then precipitated and a known amount of glucose dissolved in the filtrate. The results are consistent with the previous ones.

The last group of results shows the consequence of adding the formaldehyde to blood specimens before precipitation. As wide a range as possible of blood sugars was sought for, and in one case, a measured amount of glucose dissolved in a small amount of 0.9 per cent salt solution was added to the blood.

It will be seen that five out of six samples of the formaldehydes investigated reacted with the alkaline copper tartrate. Sample No. 4 had no appreciable influence.

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Concerning the results and conclusions presented by Denis and Aldrich, it must be inferred that an inadequate number of formaldehyde samples were investigated.

SUMMARY.

It does not seem advisable to use the average formaldehyde for the preservation of blood specimens intended for blood sugar determination.

EFFECT OF A SMALL BREAKFAST ON THE ENERGY METABOLISM OF CHILDREN.

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(Received for publication, December 7, 1923.)

It has been the custom of most workers in the field of energy metabolism to make routine observations in the morning 12 or more hours after the last meal. When a study of a large number of children in the University Elementary School was begun, however, it was found difficult to get the children to the laboratory before breakfast and early enough to permit observer and children to attend 9 o'clock classes. It was suggested that the children be allowed their usual breakfast and come for the observations during the noon hours. Most of them are at the school by 8.30 a.m. so that at least 4 to 5 hours would have elapsed since their breakfast. To determine whether we would be justified in assuming that a noon observation under these conditions was truly a basal value, we have studied the oxygen consumption of a number of children before breakfast and again on the same day 4 hours after a light breakfast.

Two points in such a study must be considered. Does the resting metabolism of a fasting individual change from 8 a.m. to 1 p.m. on the same day; and, how long does the specific dynamic action of a small breakfast continue in children?

A number of observations on the possible diurnal variation in the basal metabolism have been made. In general they show that there is a slight or no difference in the metabolism of a fasting subject.

Benedict (1) in 1915 discussed diurnal variation as a possibility in basal metabolism observations. As illustrations he cites only the case of the fasting man of the Nutrition Laboratory (2) who showed a higher metabolic rate between 7.00 and 7.45 p.m. than from 8.30 to 9.30 a.m., and many of the infants studied by Benedict and Talbot (3). A few years later, however,

Benedict and Carpenter (4) in the publication on "Food ingestion and energy transformations" collected many more observations showing absence of any diurnal variation. A number of experiments, conducted chiefly at Wesleyan University, were made with the respiration calorimeter, the periods lasting 8 or 24 hours. The subjects fasted and muscular activity was reduced to a minimum. They found that the basal metabolism at different times during a single day showed a remarkable constancy. Later, in Boston, using the respiration apparatus and 10 to 15 minute periods they studied the basal metabolism of over thirty subjects between 8 a.m. and 1 p.m., and observed no noticeable tendency toward an alteration in oxygen consumption as the day progressed. These authors therefore conclude: "Inasmuch as the average values from period to period did not tend to change in any one direction, they were evidently free from diurnal influence."

Lusk (5), quoting from Johansson, compares the carbon dioxide output and the temperature of a fasting man in complete muscular repose at different periods during the same day. He concludes that the diurnal variation in metabolism shown by the individual leading a normal life tends to disappear under basal conditions of fasting and rest.

The cases cited above, therefore, show that in most cases the basal metabolism of a fasting individual in repose tends to remain constant throughout the day.

Very little has been published concerning the specific dynamic action of a small meal and none at all concerning this action in young children. Most investigators have used single foodstuffs and have given large amounts at a time. Benedict and Carpenter (4) include only a few experiments which are in any way comparable with ours. Four may be selected, in three of which the meal consisted of milk alone and in one a usual breakfast. From 4 to 6 hours after these meals the oxygen consumption was from 0.9 to 4.0 per cent higher than the basal just before the meal.

An investigation of the specific dynamic action of food in eleven infants $2\frac{1}{2}$ to $13\frac{1}{2}$ months old made by Benedict and Talbot (3) leads to the conclusion that in most cases the minimum values for heat production were not reached before 8 or 9 hours after the feeding, except with a meal as small as 50 calories when the heat production returned to basal within 4 hours.

When studying the metabolism of boys 12 to 13 years old Du Bois (6) allowed the Boy Scouts, who acted as subjects, a breakfast of an egg, one slice of toast with butter, and one glass of milk at 7 a.m. when beginning the experiment at 11 a.m. He states that Higgins made a series of observations on a young man who took the same breakfast and found that metabolism returned to basal $3\frac{1}{2}$ hours after the meal. He gives no data for this.

Soderstrom, Barr, and Du Bois (7), impressed with the inconvenience to the patient of a delay in the breakfast hour, studied the effect of a small breakfast on the basal metabolism of five adults, making only one observation in each case. The experiment lasted from 15 minutes to 8 hours after a standard breakfast which consisted of one slice of bread, 8 gm. of butter, 10 gm. of cane-sugar, and 60 cc. of milk. They found that the average metabolism at the end of the 2nd hour was only 2 per cent above the basal rate, and at the end of the 6th hour slightly lower.

Recently Benedict and Benedict (8) have found that a breakfast consisting of 1 cup (200 cc.) of caffeine-free coffee, 16 mg. of saccharin, 30 gm. of medium cream, and 25 gm. of potato chips has no effect upon basal metabolism measurements taken an hour after the meal.

These few cases, with the exception of the infants, tend to show that a small breakfast such as a child would eat would probably have no influence on the basal metabolism some 4 hours after the meal. Because of their meagerness and occasional contradiction it seemed wise to investigate further before permitting a change in the routine hour in our observations on children.

EXPERIMENTAL. •

Seven children, five girls and two boys, between the ages of 10 and 13 years served as subjects for this investigation, with one exception all being studied two or more times. Most of the children had been shown by a medical examination to be in fairly normal health; two, Elsie and James, were distinctly underweight and in poor general condition.

In determining their basal metabolism the Benedict portable respiration apparatus was used with the general procedure as described by Blunt, Nelson, and Oleson (9) taking similar care to keep the children relaxed and quiet. As most of the children were members of the Health Class of the Home Economics Department they were accustomed to various tests. Two or more 10 minute periods were included in every observation and duplicate readings taken within each period. Only results agreeing within 5 per cent were accepted.

Immediately after the test the child was given a simple breakfast consisting of cereal, milk, bread, butter, and occasionally fruit. The protein and caloric values of the first six breakfasts were roughly estimated, but the rest were more closely determined by weighing the food which the child ate in the laboratory. The meals were all fairly uniform in character, but varied somewhat in amount. The fuel value ranged from 200 to 470 calories and the protein from 3.0 to 13.8 gm. About 3½ hours after breakfast the child returned to the laboratory, rested half an hour, and had his metabolism again determined. The children were warned particularly about not taking food in the interval and were always questioned concerning this point when they returned for the second test. On 4 days a mid-morning

TABLE I.

Comparison of Oxygen Consumption of Children Observed before Breakfast and at Noon, 4 Hours after a Small Breakfast.

Subject. Age. Weight. Height.	Date.	Breakfast.			Interval between breakfast and noon observa- tion.	Pulse.		Oxygen consumption per min.		
		Time begun.	Fuel value.	Protein.		Before breakfast.	Noon.	Before breakfast.	Noon.	Variation between noon and before- breakfast.
	<i>1922</i>	<i>a.m.</i>	<i>cal.</i>	<i>gm.</i>	<i>hrs.</i>			<i>cc.</i>	<i>cc.</i>	<i>per cent</i>
Elsie.	Apr. 22	8.30	200	3.0	4.00	106	108	165	168	1.8
12 yrs.	" 29	8.30	240	6.0	4.00			175	173	-1.2
25.2 kg.	May 13	8.30	310	4.0	4.00			172	161	-6.4
134.5 cm.	Aug. 19	8.40	395	11.0	4.00	102	102	160	167	4.3
	" 21	9.00	470	13.8	4.00	92	88	170	165	-2.9
Frances.	June 24	8.45	290	11.5	3.75	80	82	173	181	4.4
13 yrs.	" 29	8.00	325	6.8	4.50	76	76	166	163	-1.8
38.5 kg.										
149.8 cm.										
Kuma (girl).	July 11	9.15	400	12.9	3.50	90	88	185	182	-1.6
11 yrs.										
38.5 kg.										
149.8 cm.										
Franklin.	July 22	8.30	360	9.4	4.25			154	163	5.8
11 yrs.	" 27	9.00	395	11.6	4.00	70	66	160	165	3.1
27.0 kg.										
126.8 cm.										
James.	July 22	9.15	440	11.6	3.75			156	163	3.2
10 yrs.	" 29	9.00	460	11.2	3.75	80	80	157	164	4.4
29.2 kg.										
136 cm.										
Katherine.	July 29	9.00	462	12.0	4.00	72	72	140	148	6.3
11 yrs.	Aug. 3	8.15	294	7.3	4.25	76	76	144	137	-5.2
33.4 kg.	" 12	8.45	375	11.0	3.75	75	80	142	148	4.2
141.7 cm.										
Ruth.	Aug. 4	9.00	331	8.2	3.75	80	78	192	186	-3.1
12 yrs.	" 5	9.00	440	12.7	3.75	80	80	184	180	-2.2
49.2 kg.	" 7	9.00	400	11.4	3.75	82	80	192	189	-1.6
157.5 cm.										
Average.....										0.6

observation, 2 hours after the breakfast, was made as well as the usual noon test. The breakfasts on these days contained from 375 to 470 calories and from 11 to 14 gm. of protein.

The results of the series of observations are brought together in Tables I and II.

TABLE II.

Comparison of Oxygen Consumption of Children Observed before Breakfast and 2 and 4 Hours after a Small Breakfast.

Subject.	Interval between before-breakfast and later tests.		Pulse.			Oxygen consumption per min.				
	Mid-morning.	Noon.	Before breakfast.	Mid-morning.	Noon.	Before breakfast.	Mid-morning.	Noon.	Variation between mid-morning and before-breakfast.	Variation between noon and before-breakfast.
	hrs.	hrs.				cc.	cc.	cc.	per cent	per cent
Ruth.	1.75	3.50	82	88	80	192	222	189	15.6	-1.5
Katherine.	1.75	3.75	72	82	80	142	150	148	5.6	4.2
Elsie.	1.75	3.80	102	100	102	160	173	167	7.8	4.3
"	2.00	4.00	92	100	90	170	182	165	7.7	-2.9
Average.....									9.2	1.0

DISCUSSION OF RESULTS.

The differences between the children's oxygen consumption before breakfast and 4 hours afterwards are very slight, averaging only 0.6 per cent. Fifteen of the nineteen times the difference was less than 5 per cent (within the limit of error) with slight variations up or down. Two of the four others are more than 5 per cent above and two more than 5 per cent below the before-breakfast value. The highest increase for the later observations was 6.3 per cent, but a similar decrease of 6.4 per cent was observed at another time. There is no relation between these slight variations in metabolism and the differences in the breakfasts eaten. It therefore seems safe to conclude that true basal values were obtained when metabolism determinations were made 4 hours after a small breakfast.

The four tests on the children on whom mid-morning observations were made showed the usual specific dynamic effect with increases above their basal values ranging from 5.6 to 15.6 per cent.

SUMMARY.

In the effort to find whether basal metabolism determinations on children may safely be made at noon instead of before breakfast, seven children were repeatedly observed early in the morning and again on the same day about 4 hours after a small breakfast. The average oxygen consumption at the later hour was only 0.6 per cent higher than before the meal. The use of the noon hour is, therefore, to be considered safe as a routine procedure provided the breakfast does not exceed 470 calories with not more than 14 gm. of protein and is eaten at least 4 hours before the metabolism observation.

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THE AMOUNT OF AVAILABLE INSULIN IN THE PANCREAS OF DOMESTIC ANIMALS.

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Considerable knowledge is being gained concerning the physiological effect of insulin when introduced artificially into the circulation, but we know nothing of the natural factors governing insulin production and utilization. The present drastic means by which the antidiabetic substance is being removed from the pancreas allows only speculation as to the form in which it actually occurs in the living gland. A little light may be thrown on this subject by reasoning from the following two observations: During 1915-1916 Long and Fenger (1) called attention to the characteristic reaction of the pancreas. The hydrogen ion concentration was found to be nearly a constant, with a pH value running between the narrow limits of 5.5 and 5.7 in all the animals examined. Doisy, Somogyi, and Shaffer (2) have shown that insulin is precipitated (incompletely) from its solution when the pH value is between 5 and 6. We may assume, therefore, with a reasonable degree of certainty that insulin is kept at its precipitation point, and consequently in its least soluble form, while stored in the pancreas.

This investigation was carried out for the purpose of obtaining some definite information regarding the amount of insulin present in the pancreas of cattle, hogs, and sheep. The method of separating the active substance which gave us the most satisfactory results is a modification of the one outlined by Doisy, Somogyi, and Shaffer (2), as follows:

* Armour and Company are not engaged in the commercial manufacture of insulin.

5,000 gm. of finely minced fresh pancreas are macerated with a mixture of 6,000 cc. of 95 per cent specially denatured alcohol (Formula No. 30: ethyl alcohol 100 parts, methyl alcohol 10 parts), 1,500 cc. of distilled water, and 200 cc. of concentrated hydrochloric acid (sp. gr. 1.20), at room temperature for 4 hours with frequent stirring. Prolonged extraction does not increase the yield of insulin; it has a tendency to thicken the mass, which retards pressing and filtration. Strain and press the residue in a suitable power press. Reextract the press-cake with 5,000 cc. of 70 per cent alcohol for 2 hours, strain, and press the residue as before. Adjust the reaction of the combined liquids to a pH value of 2.5 by means of sodium hydroxide solution and filter. Evaporate off the alcohol *in vacuo* at a temperature below 30°C. Measure the liquid and without filtration make the volume up to 1,250 cc. with water. Add 500 gm. of granular ammonium sulfate and stir until the salt is dissolved. Filter and extract the wet precipitate with 250 cc. of 80 per cent alcohol, containing 0.14 per cent hydrochloric acid. Centrifuge, collect the clear liquid, and extract the residue once more, using the same quantity of acidulated alcohol. The precipitated insulin does not carry sufficient acid to render it entirely soluble in 80 per cent alcohol, so that it is necessary to acidify the alcohol to insure complete solution. This applies both at this step and later on in the purification process whenever insulin is being taken up in alcohol. Adjust the combined liquids to pH 5.5, add 6.5 volumes of 95 per cent alcohol, and set aside in a chill room for at least 48 hours.

Insulin is quite soluble in acidulated 93 per cent alcohol, and it is necessary to make the pH adjustment carefully and to give the slow forming precipitate ample time to flocculate. If these precautions are neglected, the yield will suffer materially.

Filter and remove most of the alcohol by evaporation. Dissolve the precipitate in 500 cc. of distilled water, containing 0.14 per cent hydrochloric acid, and filter. Add 150 gm., of granulated sodium chloride and stir until dissolved. If the precipitate does not flocculate promptly, add a little sodium hydroxide solution cautiously until a clear separation is accomplished, and filter. This second salting out removes, among other things, most of the ammonium sulfate. Take up the well drained, but still moist, precipitate in 250 cc. of 80 per cent alcohol containing 0.14 per

cent hydrochloric acid and filter. Adjust the pH to about 5.5 and add 8 volumes of 95 per cent alcohol. Allow the precipitate to stand in a chill room until the supernatant liquid is perfectly clear. This requires 2 or 3 days. Wash the precipitate with 95 per cent alcohol until the filtrate is free from chlorides and dry *in vacuo* at room temperature.

The moist insulin precipitates, whether in crude or purified form, should be exposed to the atmosphere as little as possible. If allowed to dry in the air, the product becomes gummy and assumes a dark reddish brown color. The finished product should be a white or light gray powder.

Three lots each of cattle, hog, and sheep insulin were prepared according to this method during the fall months. The glands were obtained immediately after removal from the animals while still warm, and trimmed free from fat and other adherent tissue as far as possible. They were next minced in a power hasher and then run through a Nixtamal grinder. The grinding process tears open the cell walls and facilitates extraction. Less than 1 hour was required from the time of removal until the minced glands were covered with acidulated alcohol.

The glycolytic properties of the various preparations were determined on rabbits. During the preliminary experimental work we were strongly impressed with the variance of rabbits in their response to insulin. Certain rabbits will go into convulsions and coma on very minute doses of insulin, while others exhibit remarkable resistance to this drug, depending probably on their individual glycogen reserve and their ability to utilize it. The necessity of employing a large number of animals is obvious. Albinos were found to be unsuitable for this work and were excluded. All tests were carried out at room temperature. The rabbits were kept on a bread, oat, and hay diet and received, in addition to this, a liberal ration of fresh vegetables for 3 days after insulin injections.

The administration of insulin in convulsive doses naturally is a severe shock to the system, and we found it advisable not to use the rabbits more often than twice a month. If the animals are employed at shorter intervals than this the results are apt to be very misleading.

The official insulin unit adopted by the Insulin Committee of the University of Toronto is defined as one-third the amount of material required to lower the blood sugar of a 2 kilo rabbit which has been fasting for 24 hours, from the normal level (0.118 per cent) to 0.045 per cent over a period of 5 hours (3). The task of maintaining a large number of animals weighing exactly 2 kilos presented several disadvantages, and so we employed rabbits ranging in weight from 1 to 2 kilos. We chose as a unit the amount of insulin per kilo of body weight necessary to produce convulsions and coma in from 60 to 70 per cent of rabbits weighing between 1 and 2 kilos within 5 hours. It has been our experience that the larger rabbits respond just as promptly to their quota of insulin as do the smaller ones and that the convulsive dose of insulin is in direct proportion to the body weight within these weight ranges. This confirms Sansum's (4) findings. That our unit is considerably larger than the official one was conclusively shown by the control tests which were invariably carried along with every set of determinations. As controls we employed several different lots of Eli Lilly and Company'sletin solution (both the H and the U varieties). For practical reasons we did not run three blood sugar determinations on each rabbit employed in the standardization work of these samples. A few tests were made regularly to ascertain that the rabbits were normal during the preliminary and routine testing; it was then considered sufficient evidence if the required number of animals went into convulsions and recovered promptly when dextrose was administered subcutaneously. A sufficiently large number of blood sugar tests was carried out in the decisive and final tests to convince us that the blood sugar content of the rabbits was normal before injection and that the convulsive and comatose stages were actually due to hypoglycemia.

The preparations intended for tests were made by dissolving the powdered insulin in isotonic sodium chloride solution, containing 0.3 per cent of acetic acid. The solutions were sterilized by heat in small cotton-plugged Erlenmeyer flasks and cooled before use. In some instances the liquids were filled into ampules and sterilized by immersing the ampules in boiling water for 15 minutes on 3 consecutive days. Control experiments showed that sterilization did not alter the physiological activity of the product

and that insulin solution may therefore be sterilized with impunity. Each lot of insulin made was tested repeatedly on rabbits, using a wide range of dosage, from 1 mg. down to one-twentieth of a milligram per kilo of body weight. This gave us the approximate strength. We then proceeded to inject rabbits of evenly distributed weight ranges in batches of ten, each lot receiving a certain dose, such as, for instance, 0.5 mg. per kilo of body weight. A 1,000 gm. rabbit would then get 0.5 mg., a 1,500 gm. rabbit 0.75 mg., and a 2,000 gm. rabbit 1 mg., etc. The next batch would receive 0.4 mg. per kilo of body weight, and so on. After several tests on different days we were able to reach definite conclusions regarding the actual strength of the samples. These purified products have not been tested clinically on human beings, but no untoward symptoms or toxic after effects were observed in our experimental animals which received repeated injections.

It will be seen from the data in Table I that while considerable variation exists between the amount and physiological activity of the small individual lots of purified insulin, the average number of rabbit units per kilo of fresh glands is in the neighborhood of 1,800 for all three species of animals. The amount of purified insulin representing 1 rabbit unit varied from 0.3 to 0.6 mg.

The number of domestic animals slaughtered yearly in the United States under federal government inspection approximates $8\frac{1}{2}$ million cattle, 4 million calves, 11 million sheep and lambs, and 43 million hogs. These animals furnish about $5\frac{1}{2}$ million kilos of fresh pancreas glands. The available supply of raw material is, therefore, far in excess of any possible demand for insulin.

The following experiment gives some interesting information regarding the solubility behavior of insulin. 100 mg. of purified beef insulin representing 333 rabbit units were ground in a mortar with 10 cc. of neutral distilled water. Only a portion of the powder went into solution. The reaction of the mixture was slightly acid to litmus. Complete solution was accomplished by the addition of 0.05 N hydrochloric acid. Litmus neutrality was then produced by adding 0.05 N sodium hydroxidé. The amount of caustic was in excess of that of acid, indicating the presence of adsorbed acid in the insulin powder. The neutralizing produced a heavy precipitate. This was separated by filtration and redissolved in distilled water by means of a little hydrochloric acid.

Tests on rabbits showed that this fraction contained 94 per cent of the total physiological activity of the original sample. The neutral clear filtrate was divided into two portions, one of which was boiled for 2 minutes. Both solutions were found to be of approximately equal activity, but constituted, combined, only 20 rabbit units, or 6 per cent of the strength of the purified product, *i.e.* 0.006 gm., which represents the amount of insulin soluble in 10 cc. of water at litmus neutrality. This amount is small from a sol-

TABLE I.

	Yield of insulin per kilo of fresh pancreas glands.	Amount of insulin per kilo of body weight required to produce convulsions and coma in rabbits.	Total No. of rabbit units per kilo of fresh glands.	Average weight of trimmed fresh pancreas glands.
	<i>mg.</i>	<i>mg.</i>		<i>gm.</i>
Cattle.	450	0 3	1,500	
	500	0.3	1,665	
	875	0.4	2,190	
Average for cattle..	610	0.33	1,785	308.0
Hog.	950	0 5	1,900	
	750	0.45	1,665	
	900	0.55	1,635	
Average for hog....	865	0.5	1,730	60.0
Sheep.	950	0.5	1,900	
	700	0 4	1,750	
	1,120	0 6	1,865	
Average for sheep..	925	0.5	1,838	18.8

ubility standpoint, but of some consequence when the potency of the product is taken into consideration. The test points out clearly the absolute necessity of having a sufficient amount of acid present whenever insulin is to be brought into solution.

As to the chemistry of insulin itself, we may say that, although it has not yet been isolated in pure form, there seems to be no reason to doubt that the substance is of a protein nature. At present we are unable to tell the exact strength of the pure substance. It must, however, be very potent. Preparations refined

to the point where 0.1 mg. of dry substance represents 1 rabbit unit still contain a high percentage of ash and are consequently far from pure. The work and experiments conducted in this laboratory indicate that insulin is a derived and not a native protein. The most active products at our disposal possess the physical and chemical characteristics of complex protein derivatives not lower than the primary proteoses.

DISCUSSION.

We have found that the most important factor in insulin production is absolutely fresh glands. It is also advantageous to break up the cell walls as thoroughly as possible in order to release the insulin and render it available for extraction. Mincing the glands in a meat chopper is not quite sufficient. The mincing should be followed by a brief but efficient grinding process. In order to prevent or minimize the action of trypsin the disintegrating process should be made continuous. The glands may be conveyed from the chopper directly into the grinder and from there right into the acidulated alcohol and water mixture. It is better to bring the glands into process while fresh and still warm than to chill them first. Freezing does much harm because the ice crystals break open the cell walls with consequent infiltration and just as soon as thawing sets in the proteolytic enzyme takes effect immediately with very disastrous results to the insulin. It is essential in all stages of the purification processes to remember that it requires the presence of acid to bring insulin into either water or alcohol solution. We are not in a position to make any definite statement regarding the keeping qualities of the dry insulin. Our samples, however, have shown no decrease in strength over a period of several months.

CONCLUSIONS.

The amount of insulin in the pancreas of domestic animals varies from 1,500 to 2,200 rabbit units per kilo of fresh glands. The average yield approximates 1,800 units from cattle, hogs, and sheep. The methods employed in separating and standardizing the purified insulin are described in detail. Some of the physical and chemical characteristics of the purified insulin are pointed out.

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OBSERVATIONS ON THE PERSISTENCE OF VITAMIN C IN THE LIVERS OF RATS ON A SCORBUTIC RATION.*

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When McCollum and Pitz (1) were led to advance the theory that scurvy is a disease having its origin in perverted intestinal conditions rather than a lack of a specific dietary constituent they were in part misled by the fact that rats can grow normally on many rations which soon prove fatal to the guinea pig. To them it appeared more probable that the anatomical structure of the cecum and colon of the guinea pig demanded a ration of particular bulk to facilitate ready elimination rather than that the rat possessed vitamin requirements different from those of the guinea pig or man. The disproof of their theory is now a matter of history, but the interesting fact that the rat can get along on less vitamin C than the guinea pig, or possibly without any in its ration presents many interesting experimental possibilities.

Harden and Zilva (2) and Drummond (3) both subscribed to the idea that the rat needs vitamin C because they observed that the juice of citrus fruits used as a supplement to purified synthetic rat rations gave increased growth. This criterion, applied to their limited experimental data, has been aptly criticized by Osborne and Mendel (4) who pointed out that additional amounts of vitamin B known to be contained in these juices might have brought about the same result. There is no doubt much justice in this criticism for as Osborne and Mendel state:

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"Without a demonstration that more yeast, which is not regarded as antiscorbutic, or some other source of water-soluble B should fail to accomplish what is claimed for orange juice added to yeast, the conclusion of the need of some special antiscorbutic by rats is not convincing."

It might be pointed out that the same is true for the fat-soluble vitamins also known to be contained in the citrus fruits. When fed in increasingly large amounts, they make possible more and more growth to a degree which makes it difficult to decide just where "normal" growth lies.

Parsons (5) also subscribes to the idea that Harden and Zilva, and Drummond have not proved their point and at the same time brings out the interesting fact that when rats are raised on a ration which at the most can contain but small amounts of vitamin C their livers still contain an abundance of this substance. She suggests a number of possibilities: Either the rat may need less of this vitamin or it may be able to synthesize it or it may even be able to utilize a form of it, present in the ration, but not available to the guinea pig.

She fed her rats a ration of cooked soy bean meal 76, NaCl 3, Ca lactate 3, yeast 3, butter fat 5, dried skimmed milk 8, and filter paper 2. The soy bean meal fed had previously been heated in an autoclave until destruction of its raw taste had just been brought about without causing marked discoloration. This was done to destroy such amounts of vitamin C as might be contained in the meal, without sacrifice of its appetizing qualities. As a further precaution the skimmed milk was omitted from the ration of the rats from 39 to 73 days before they were sacrificed for their livers. It also was not fed at the beginning of the experiment.

The rats used by Parsons were started on the above ration when about one-third grown and continued on it from 213 to 247 days. Livers of these rats in 5 gm. daily portions were found as efficient in curing scurvy in guinea pigs as those taken from rats receiving a stock ration supplemented with 5 cc. of orange juice daily.

These results appeared to us too important from the standpoint of other investigations on the antiscorbutic vitamin from this laboratory to be left unconfirmed. We have accordingly duplicated the work of Parsons, using what we consider an improved vitamin

C-free ration on which the rats were kept from the time of weaning, and in addition we have used second generation rats raised on this ration for the source of liver in a second series of experiments.

EXPERIMENTAL.

The ration fed our rats was composed of yellow corn 80, flax-seed oil meal 13.5, casein 5, sodium chloride 0.5, bone ash or calcium phosphate 2, and the unsaponifiable fraction obtained from 2.5 to 5 parts of cod liver oil. The constituents exclusive of the cod liver oil fraction were thoroughly ground and mixed, then moistened with water, and autoclaved at 15 pounds pressure for 1 hour. After drying at 70–80° for 1 week the material was again ground and an ether solution of the required amount of cod liver oil preparation poured on it. This was prepared as previously described (6), but with only one saponification.

We found this ration very good for supporting growth in our rats although it was not found as satisfactory as our stock ration; reproduction in the second generation was markedly interfered with. On account of the drastic heat treatment we rather suspected a vitamin B deficiency, but in a few test animals the addition of 5 per cent yeast did not cause distinct improvement. Results in a limited number of trials with additions of cod liver oil, or orange juice, or yeast combined with cod liver oil and orange juice did not solve the difficulty. We are convinced that the trouble, at least in part, was due to changes incidental to the heating and drying operations because when young rats produced by mothers on the heated ration were in turn put on the unheated ration they not only grew better but gave birth to and even reared some young. In the same interim similar young kept on the heated ration failed to become pregnant (Chart I).

We were here only concerned incidentally with the failure of normal growth and reproduction as we feared that we would not succeed in raising a sufficient number of animals for our second generation experiments. Our fears proved unfounded so we did not investigate this matter further. Our results did show us, however, that our rat ration had been heated dangerously near the limit where continued production became impossible.

The rats were started soon after weaning on the experimental ration—their weights ranging from 48 to 74 gm., average 56 gm. They were fed the ration *ad libitum*, six in a cage measuring 2 feet square, and the cages were bedded with shavings. Distilled water was provided from drinking tubes. They were sacrificed for their livers when they weighed from 220 to 330 gm., average 279 gm. The interval spent on the ration ranged from 103 to 114 days, average 108 days. In the second generation experiments no accurate record of ages and weights was kept. Though their

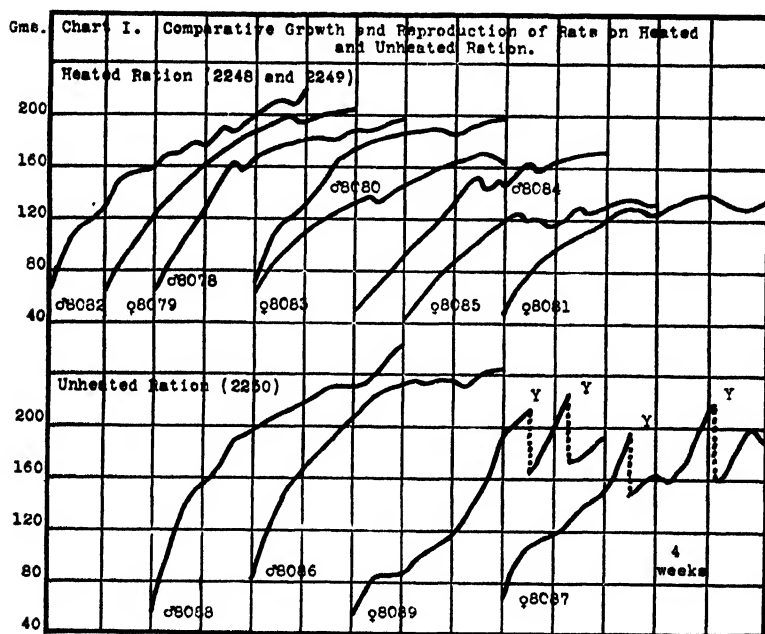


CHART I. Shows the effect of heating a grain ration on growth and reproduction in rats. A grain ration to the exclusion of milk or alfalfa supplements was used as grains are admittedly low in their content of vitamin C. This was heated in an attempt to reduce further the vitamin C content. As the chart shows this turned out to be prejudicial to the well-being of the rats. Rats 8078, 8079, 8083, 8086, and 8087 were litter mates and so were Rats 8080, 8081, 8082, 8084, 8085, 8088, and 8089. They represent young raised by mothers which had received the heated ration from the time of weaning. Rat 8087 raised both litters of young, but those of 8089 disappeared soon after birth.

growth was slow all rats were in good condition with no obvious signs of malnutrition when sacrificed.

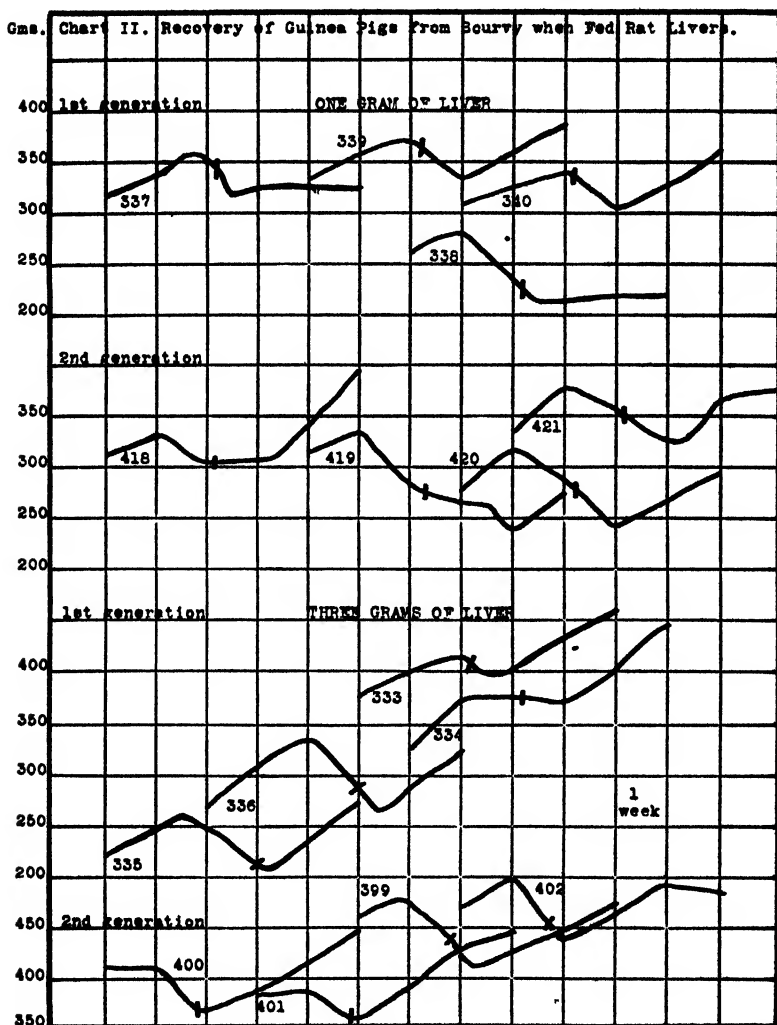


CHART II. The daily administration of 1 or 3 gm. of rat liver obtained from rats raised on a heated grain ration brought about rapid recovery of scorbutic guinea pigs. Livers of second generation rats raised on this ration were as efficient as those of the first.

The guinea pigs used for the tests were fed our standard scurvy-producing ration (7) for 12 to 21 days depending upon the time and severity of incidence of scurvy. This was determined by their fall in weight and degree of involvement of the wrists. When all question of failure from scurvy was removed liver as prepared by Parsons was administered in graduated amounts.

Chart II shows the results obtained when liver from first and second generation rats was fed daily in 1 and 3 gm. quantities to the scorbutic pigs. It is obvious that no difference obtained. Recovery in all cases except Guinea Pig 419 was fairly prompt. Recovery was measured not only by increase in weight but renewal of normal activity and subsidence of swelling of wrists or other detectable involvements. Usually the guinea pig receiving 1 gm. of liver became more active after 7 or more days of liver administration. When getting 3 gm. of liver the same resulted usually in 3 to 4 days. Increase in amount of liver is marked not so much by increased resultant gain as by the promptness with which increase in weight resulted.

Acknowledgement must be made Professors Steenbock and Hart for their guidance and to them we wish to tender our sincere thanks.

SUMMARY.

Young rats taken soon after weaning and put on a heated grain ration supplemented with salts and additional fat-soluble vitamins after 114 days still contain an abundance of vitamin C in their livers.

A second generation of rats was raised with difficulty on this ration yet their livers were as rich in vitamin C as those of the first generation.

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SOME FURTHER OBSERVATIONS CONCERNING THE ANTISCORBUTIC REQUIREMENT OF THE RAT.*

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INTRODUCTION.

Investigations concerning the vitamin requirement of different species of animals and of man have revealed in a number of instances, a fundamental similarity among animal cells in this regard. Indeed, so similar is the demand for antiscorbutic vitamin on the part of man and the guinea pig, for instance, that the size of effective doses of antiscorbutic food for infants can be determined by tests with the guinea pig almost in the manner of an assay. Such similarity in requirement, however, makes all the more striking certain well established exceptions to this principle. One of the most remarkable of these exceptions is the dissimilarity in the antiscorbutic requirement of the rat and the guinea pig, two animals very closely related in the biological classification.

The fact that diets containing no known source of antiscorbutic vitamin have been successful in nourishing the experimental rat has led to some doubt as to whether this vitamin has any place in the metabolism of the rat, or is perhaps synthesized by this animal. On the other hand, Harden and Zilva (1) and Drummond (2) noted a certain degree of improvement in the growth and reproduction of groups of rats to whose diet antiscorbutic vitamin was added, in comparison with control groups. Drummond, therefore, concluded that the antiscorbutic requirement of rats,

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Based upon graduate work of the junior author performed in the Department of Home Economics in 1920-1921.

although small, does not differ fundamentally from other types of higher animals.

In view of the meager data on this fundamental problem it seemed important to one of us (H. T. P.) to determine whether or not the antiscorbutic vitamin is present in the tissues of the rat; if so, to what extent; and what influence the concentration of this vitamin in the diet would have on its concentration in the rat's tissues. The results of this investigation (3) showed a high content of antiscorbutic vitamin in the liver of the rat, and gave no evidence of any variation in its concentration in this tissue due to variations in the diet of the rat from a ration high in orange juice on the one hand, to a typical scorbutic ration on the other. These results were of such theoretical interest that the report of this investigation included a suggestion of the desirability of making an attempt to eliminate traces of antiscorbutic vitamin in the scorbutic ration even more thoroughly than had been done in this instance, as well as prolonging the interval during which the rats were restricted to the ration.

Since the study had involved a comparison between the requirement of the rat and the guinea pig, the modified soy bean ration of Givens and Cohen (4) which was being fed at that time for the production of scurvy in the latter animal was used as the low antiscorbutic diet in feeding the rat in this first investigation.

Soy Bean Scorbutic Ration I.

	<i>per cent</i>
Autoclaved soy bean meal	76.0
NaCl	3.0
Ca lactate	3.0
Dried yeast	3.0
Butter fat	5.0
Dried skimmed milk	8.0
Filter paper	2.0

It was considered that this ration was no more questionable in regard to the possibility of its containing significant traces of antiscorbutic vitamin not demonstrable by the guinea pig's high requirement, than another diet which was also considered; *i.e.*, the standard purified ration for the rat in use in the laboratory. In the latter, vitamin B was added in the form of an alcoholic extract, which might possibly have contained traces of the antiscorbutic vitamin, as alcohol is also an excellent solvent for this vitamin. Dried milk was at first included in the scorbutic ration, according to the formula of Givens and Cohen (4). At this time

there was considerable confusion in the literature concerning the antiscorbutic content of dried milk, since it had not yet been made clear that variations occur in this product due to conditions under which it is produced. A report (5) of the virtual absence of this vitamin from commercial dried milk gave confidence that at most only insignificant traces of antiscorbutic vitamin would be included in the 8 per cent of dried milk in the soy bean ration. Later, however, when other reports (6, 7) were read which made apparent the possibility of this product's furnishing a considerable source of the antiscorbutic vitamin, dried milk was omitted. 39 to 73 days elapsed after its withdrawal from the ration (the variation due to the time of killing the animal) before the livers of the animals were fed to scorbutic guinea pigs.

It was for the purpose of prolonging the interval of feeding the scorbutic ration to the rat and of securing a more nearly antiscorbutic-free ration by means of suitable selection, purification, or heat treatment of the food materials that the present investigation was undertaken.

EXPERIMENTAL.

Animals.—Three generations of rats were fed the purified scorbutic diet. The first generation rats, however, were fed only from the time that they were mated for the production of the second generation. They were discarded after weaning the second generation young and their livers were not fed. The livers of the second generation and the third generation rats were the ones tested for their antiscorbutic content.

Ration.—Because of the tediousness of preparing and drying dextrin, the following high fat rations were used so that raw starch could be incorporated in them. These rations differ only in their content of wheat embryo.

Purified Scorbutic Rations.

Ration No.....	I	II	III	IV
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Casein.....	20	20	20	20
Starch.....	53	51	45	43
Butter fat.....	20	20	20	20
Salt mixture 185.....	5	5	5	5
Wheat embryo.....	2	4	10	12
Total.....	100	100	100	100

The first generation rats were started on Purified Ration I containing 2 per cent wheat embryo. As soon as pregnancy was detected in the females, the wheat embryo was raised to 10 per cent in their diets (Ration III) to take care of the extra demand for this vitamin. The first litter of the second generation rats was kept for a time on 2 per cent and then 4 per cent wheat embryo (Rations I and II), but as their growth was somewhat slow, 10 per cent wheat embryo was continued in their ration as well as in the ration of all succeeding litters of this generation. The third generation litters were given 12 per cent wheat embryo (Ration IV).

The methods of purifying the various constituents contained in the rations follow.

The Argo corn-starch was purified by two extractions, with 95 per cent alcohol, each a day in length. The alcohol was filtered off by means of a suction filter and the starch spread out to dry. Commercial casein was prepared in the same way for feeding the first and second generation rats. For the third generation, the added precaution was taken of dialyzing the casein with two changes of 0.2 per cent glacial acetic acid solution for 2 days. The mass was stirred occasionally, was finally washed, and dried and treated with alcohol as in the previous preparation.

The salt mixture was No. 185 used by McCollum and Simmonds (8).

	gm.
NaCl.....	17.3
MgSO ₄ (anhydrous).....	26.6
NaH ₂ PO ₄ ·H ₂ O.....	34.7
K ₂ HPO ₄	95.4
CaH ₄ (PO ₄) ₂ ·H ₂ O.....	54.0
Fe citrate.....	11.8
Ca lactate.....	120.0

The portions of the salts were weighed, ground, and mixed together after a prolonged period of heating to render them suitable for grinding.

The wheat embryo was prepared after the method used by Daniels and McClurg (9) in treating navy and soy beans. It was made slightly alkaline by adding 33 cc. of a 5 per cent solution of sodium bicarbonate for every 50 gm. of wheat embryo. The mixture was boiled 1 hour. The excess alkali was then neutralized with hydrochloric acid and the material evaporated to

dryness on a sand bath and ground. The somewhat subnormal growth of the young on the rations containing low percentages of wheat embryo was thought to have been due to a possible destruction of some of vitamin B by this alkali and heat treatment.

The rats of the first generation persisted in eating the shavings of the bedding and as it is not known that they are free from antiscorbutic vitamin, the shavings were boiled for 1 hour in a 2 per cent solution of sodium hydroxide. They were left under running tap water overnight to wash them free from the alkali and then spread out to dry. After using the treated shavings for 2 weeks, white crepe paper waste was obtained. This material was not treated in any way as it was considered that all antiscorbutic material had been destroyed in the process of manufacture.

The scorbutic ration fed to the guinea pigs for the production of scurvy was the following one, used in the previous investigation (3).

Soy Bean Ration II.

	<i>per cent</i>
Soy bean meal (Hepco)	84.0
Ca lactate	3.0
Dried yeast	3.0
NaCl	3.0
Butter fat	5.0
Filter paper	2.0

The soy bean meal was heated in a pressure cooker at 15 pounds for 10 minutes, as this takes away the raw taste which the guinea pigs dislike. The butter fat was prepared in the same way as in the purified ration. A weighed amount of filter paper was further broken up by beating it with a Dover egg beater. The soy bean meal was mixed thoroughly with the wet filter paper and more distilled water was added as needed. The butter fat was melted and all other ingredients were added and mixed. The thick mass was then spread in thin layers on large, shallow pans and dried over a steam radiator. The mass was turned occasionally so that it dried evenly.

Preparation of the Liver.—The rat which was to furnish the liver to be fed to the guinea pigs was chloroformed. As soon as the animal stopped breathing, the liver was removed. The amount to be fed was weighed out on the analytical balance. The liver was then cut into small pieces and rubbed through a sieve. The material which adhered to the sieve was washed off

TABLE I.

Serial No. of guinea pigs.	No. of rats used.	Age of rats used. days	Ration of rats.	Generation of rats on ration.	Ration of guinea pigs.	Weight of rats liver-fed daily.	Day of experiment on which addition began.	Day on which scurvy symptoms were most severe.	No. of days the addition was continued.	Remarks.
IX	46	80-106	Scorbutic III.	1st	Soy bean.	7	14	16	24	Scurvy symptoms disappeared on addition of liver. Chloroformed on 39th day.
X	46	80-106	" III.	1st	"	3	14	17	20	Scurvy symptoms disappeared on adding liver. Death occurred on 35th day.*
XI	31	51-135	" III.	1st	"	3	14	16	24	Symptoms of scurvy disappeared on adding liver. Chloroformed on 38th day.
XII	31	51-135	" III.	1st	"	2	14	18	24	Scurvy symptoms disappeared on adding liver. Chloroformed on 38th day.
XIII	8	152-164	" IV.	2nd	"	5	12	13	12	Scurvy symptoms improved on adding liver. Chloroformed on 25th day.

* Death occurred on the 35th day while the guinea pig was being fed. The animal appeared in vigorous health. The cause of death was probably choking.

with distilled water into the container. Enough distilled water was added to the strained liver to make a good consistency to feed to the guinea pigs with a medicine dropper. The greatest care was exercised to have as quantitatively accurate methods of preparing and feeding the liver as possible. The livers of two rats usually furnished the amounts needed for two guinea pigs. The liver was fed to the guinea pigs once daily and they consumed it greedily.

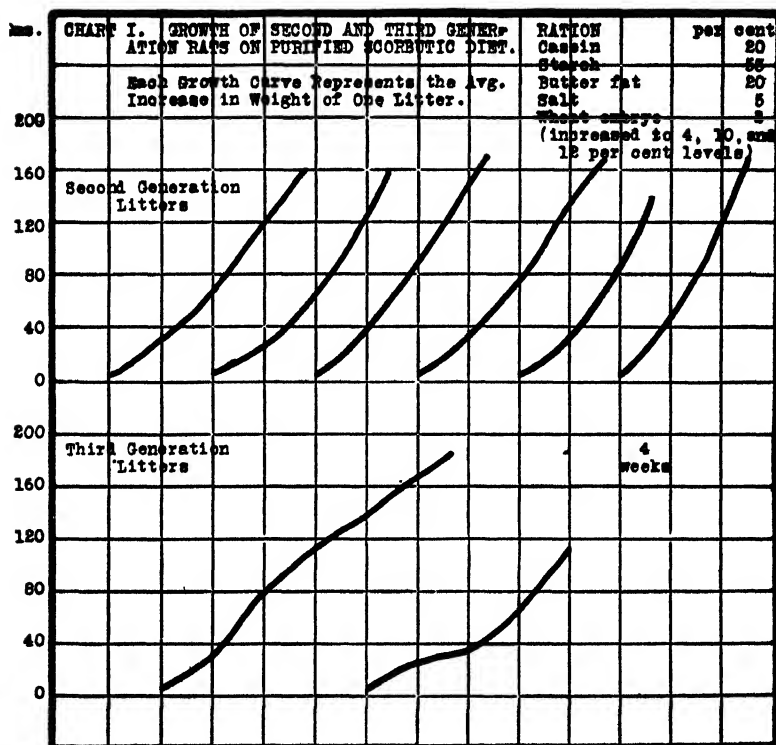


CHART I.

RESULTS.

The results of the experiment are recorded in Table I and in Charts I and II. It will be seen that, in the amounts fed, there is no perceptible decrease in the efficiency of the doses of

rat liver to relieve the symptoms of scurvy in the guinea pig, even though the rats have been restricted for two complete generations to a purified ration presumably containing at most only traces of the antiscorbutic vitamin.

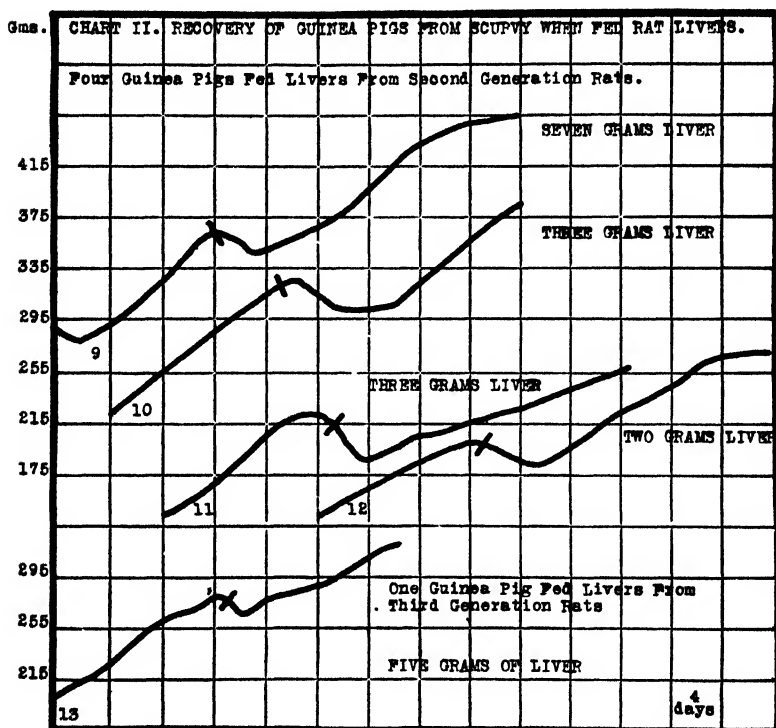


CHART II.

DISCUSSION.

Among the various hypotheses to account for the marked persistence of the antiscorbutic vitamin in the liver of the rat suggested and not fully proved or disproved in the previous report (3) one is in substance as follows: the rat has a very definite food requirement for the antiscorbutic factor, but this is quantitatively very small in comparison with the requirement of the guinea pig and can be met by certain diets very low in this factor on which the guinea pig acquires pronounced scurvy. This hypothesis is

rendered less probable by the present investigation, since two successive generations of rats have been produced and reared upon a carefully purified ration without noticeable diminution in the concentration of the antiscorbutic factor in their livers.

Other hypotheses will be discussed in further reports of investigations on this problem.

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DIURNAL VARIATIONS IN THE RATE OF URINE EXCRETION FOR TWO HOUR INTERVALS: SOME ASSOCIATED FACTORS.

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It is common knowledge that less urine is excreted during the night than during the day and that in nephritis the curve of urine volume is flattened. It has not been determined to what extent normal variations are due to decreased activity and decreased ingestion of food and water during the night. Knowledge of variations in volume and concomitant changes in composition of the urine for short intervals should be of interest *per se* and, moreover, might be expected to yield information regarding the process of water excretion by the kidneys and the part the tissues play in determining this, if any. The results of such a study should serve as a base line for the study of edema and the effects of various salts, etc., on water elimination—a field which is in a notoriously unsatisfactory condition.

Reports in the literature showing urine volumes for short intervals during 12 or 16 hours during the day, usually incidental to metabolism studies, are numerous. They show wide variations and little regularity even when constant amounts of water are ingested (see Neuwirth (1), for instance). Rosemann (2), in a study of nitrogen excretion, recorded a large number of 2 hour urine volumes, usually for 16 hours during the day, when meals were taken, and once for 36 hours without food. Neuwirth and Rosemann engaged in laboratory work during their experiments. These workers, and many others, show water excretion at its maximum in the morning, usually, and, oftentimes, a second maximum point between 6 and 9 p.m.

In studying urine volume and its regulation, certain conditions should be observed, at least until it is shown that they are not essential. It is first of all imperative that physical activity be limited. If water and food are given, the intervals should be so short that any flooding effect is ruled out. Wide variations in temperature and humidity should be excluded.

EXPERIMENTAL PART.

The carrying out of these experiments has been made possible by the cooperation of a large number of medical students, to whom the writer is indebted.¹ Most of the men engaged in some form of university games or athletics during the year. The subject came to the laboratory before midnight, received an enema, and retired. In the morning he was awakened between 6 and 9 a.m. Thereafter temperature and humidity of the room were regulated. The air in the room was kept in motion by a fan which was directed away from the subject and toward the wet bulb of the hygrometer.² The pulse and temperature of the subject were taken in the morning on waking. He then voided, was weighed (when scales were available), and received a measured amount of water — at 38–40° in the earlier experiments. Later it was found that when 100 cc. of water were given results were not different if the water was taken from the distilled water tank in the room, that is, at about room temperature. Pulse and sublingual temperature observations were made, and water was given, every hour for 24 or more hours. Every 2 hours the subject stood beside the bed to urinate and to be weighed. Defecation was unnecessary during the experimental period. The maximum activity was reading in bed, except as indicated above. Subjects were selected who claimed to be good sleepers, a claim that was well borne out since a normal amount of sleep was obtained in all but one

¹ The data presented and the discussion accompanying are based on sixteen experiments, as follows:

Four experiments: 100 cc. of water at 38°C.; total N, uric acid, creatinine, and chlorides; ammonia and urea in one of these.

Three experiments: 100 cc. of H₂O at room temperature; no analyses.

Four experiments: 100 cc. of H₂O at room temperature during the day, an additional 100 cc. when the retention period began; ammonia and urea N.

Three experiments: 200 cc. of H₂O at room temperature; uric acid, creatinine, chlorides, ammonia, and urea; pH and titratable acidity in two of these.

Two experiments: 10 gm. of glucose plus 200 cc. of H₂O at 38°C.; pH, ammonia, and urea; titratable acidity in one of these.

² This work was begun in midwinter. In the spring humidity and temperature were merely recorded, since variations were small. It was perhaps fortunate that almost all experiments were carried out on rainy days.

experiment.³ No endeavor was made to limit sleep to certain periods, but if the subject was not asleep by 11 p.m. (and had not slept to any extent during the day) sleep was induced by reading to him in a monotonous tone. At night, after the subject fell asleep, the clinical thermometer was introduced under the tongue with the subject hardly conscious. He was handed the water in the dark, and usually dropped off to sleep without delay.

Standard methods were used in the analyses (3-7). Titratable acidity and pH determinations were completed by noon of the day on which the experiment ended, uric acid and creatinine usually by the evening of this day, ammonia by noon of the following day, and urea on the day after this. In many of the 100 cc. experiments urea determinations were necessarily delayed and total nitrogen was determined instead. pH was determined colorimetrically using brom-phenol blue, phenol red, and methyl red. A few of the standard solutions were checked with the potentiometer.

Results in the tables and charts are expressed in units excreted per hour for 2 hour intervals, unless otherwise stated.

RESULTS.

A. Experiments with 100 Cc. of Water Hourly. See Chart 1.

As the body temperature rises in the morning there is a sharp increase in water excretion with a negative water balance as long as the temperature is elevated. This rise is not associated with bodily activity or with waking from sleep. In many experiments the subject, after waking, lay perfectly still. But the rise in temperature and urine volume occurred, nevertheless. In Experiment C, Chart 1, the subject was awake from 6 a.m., but the increase in temperature and volume did not occur until noon.

When temperature begins to fall—and this may occur anywhere from 4 to 10 p.m.—there is a sharp drop in volume. A

³ G. E. S. was the subject in two experiments, in one of which (2) the distilled water was placed beside the bed and the subject was not waited on during the night. Rosemann, who carried out his experiments on himself in this way, noted that under such conditions a normal amount of sleep was not obtained. This is true. In another experiment on G. E. S., when he was waited on, as were the subjects of all the other experiments, a normal amount of sleep was obtained.

stage of water retention sets in, with volumes as low as 12 or 13 cc. an hour. The onset of this is not associated with sleep, necessarily. It lasts until about 7 a.m., when there is a sharp

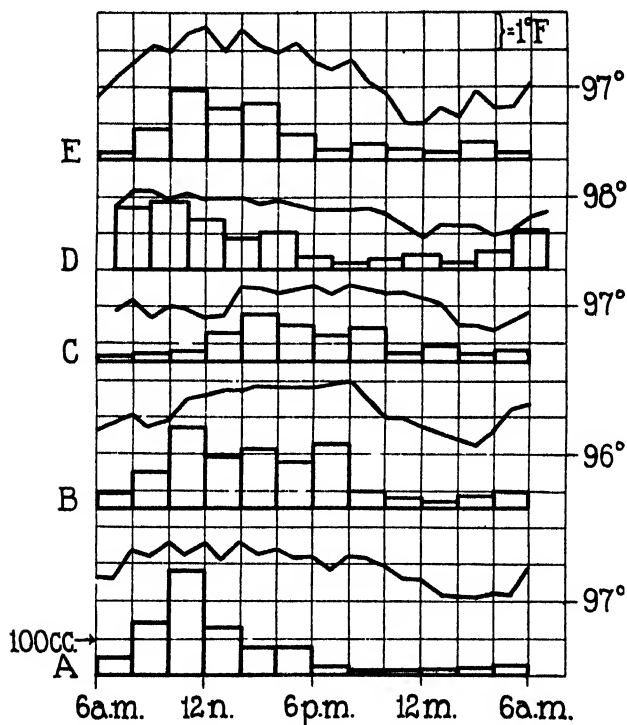


CHART 1. Urine volume (blocked) and body temperature (curve), when 100 cc. of water are given hourly—at 38°, in Experiments A to D; at room temperature in Experiment E.

Experiment A. Room temperature 70–72°. Humidity 57 to 59 per cent. Subject B. R. Mar. 10, 1923.

Experiment B. Room temperature 70–72°. Humidity 44 to 46 per cent. Subject K. S. M. Mar. 20, 1923.

Experiment C. Room temperature 68–71°. Humidity 44 to 50 per cent. Subject W. H. J. A. Feb. 9, 1923. Rise in temperature and increase in volume later than usual.

Experiment D. Room temperature 70–72°. Humidity 37 to 44 per cent. Subject G. E. S.-1. Mar. 29, 1923.

Experiment E. Room temperature 64–70°. Humidity 40 to 66 per cent. Subject G. C. P. May 15, 1923.

TABLE I.
Effect of Water Retention on Excretion of Creatinine and Urea.

Experiment No. and amount of water.	Length of time. hrs.	Time.	Volume.		Creatinine.		Urea.		Remarks.
			cc.	per cent	mg.	per cent	mg.	per cent	
1 100 cc. H ₂ O.	8	2 p.m.-10 p.m.	98	100*	91	100	589	100	Sleep.
	8	10 " - 6 a.m.	28	29	78	86	315	53	
2 100 cc. H ₂ O.	6	11 a.m.- 5 p.m.	107	486	70	101	314	162	Sleep.
	6	5 p.m.-11 "	22	100	69	100	194	100	
	6	11 " - 5 a.m.	28	127	72	104	293	151	
3 100 cc. H ₂ O to 2 a.m., 200 cc. thereafter.	4	4 p.m.- 8 p.m.	68	100	75	100	369	100	Sleep.
	6	8 " - 2 a.m.	49	72	71	95	345	93	
	4	2 a.m.- 6 "	166	244	75	100	480	130	
4 200 cc. H ₂ O.	4	9 a.m.- 1 p.m.	192	86	74	104	383	112	Sleep.
	8	1 p.m.- 9 "	224	100	71	100	343	100	
	8	9 " - 5 a.m.	110	49	62	87	274	80	
	4	5 a.m.- 9 "	222	100	71	100	400	116	
5 200 cc. H ₂ O.	8	5 a.m.- 1 p.m.	310	155	74	114	645	158	Sleep.
	8	1 p.m.- 9 "	200	100	65	100	409	100	
	8	9 " - 5 a.m.	176	88	67	103	358	87	
6 200 cc. H ₂ O.	8	1 p.m.- 9 p.m.	201	100	52	100	239	100	Sleep.
	8	9 " - 5 a.m.	163	81	53	102	189	79	
	8	5 a.m.- 1 p.m.	192	95	53	102	296	124	

rise in temperature while the volume increases, usually a little later. (As many of the experiments ended at 6 a.m., this secondary rise was sometimes missed. It was seen in all experiments lasting beyond 7 a.m.) There is no proportionality between body temperature and urine volume. Indeed, after the temperature begins to fall it may decrease markedly with no further volume decrease.

The excretion of urea or total nitrogen follows the curve of volume more or less closely. The amount of urea excreted during the height of water elimination in the day may be three times that of the night, when water is retained (Table I, Experiments 1, 2, and 3, shows the night retention); the concentration is greatest during water retention. Since one of the objects of this work was to determine to what extent and under what conditions, for short intervals, the composition of the urine can be regarded as an index of metabolism, the water allowance was increased to 200 cc.

B. Experiments with 200 Cc. of Water Hourly.

One of these experiments was carried out in the dark room, in order to determine if absence of light would affect the curve. The results were not different from the other experiments, one of which is shown in full in Chart 2. Part of the data for these experiments are given in Table II, and part in Table III. In all these experiments the subject was weighed every 2 hours on scales sensitive to 10 gm., in order to determine if, perhaps, a large amount of water was excreted by other channels during the time when little was being excreted by the kidneys. The data from six experiments thus far completed do not show any relationship. By "insensible perspiration" in Chart 2 is meant the loss in body weight not accounted for by the weight of the urine.

The decrease in urea excretion during the night (Experiments 4, 5, and 6, Table I) is now on the order of what would be expected on the basis of the lowered metabolism, which, as Benedict (8) has shown, is about 80 per cent lower during sleep than in "sitting awake." Table I (Experiments 4 and 6) shows that more urea is excreted after waking on the 2nd morning than before the retention period set in on the previous day, so that urea may

have been retained during the night periods even with the larger allowance of water. However, it would be expected that the amount of urea would be greater on the 2nd day, due to

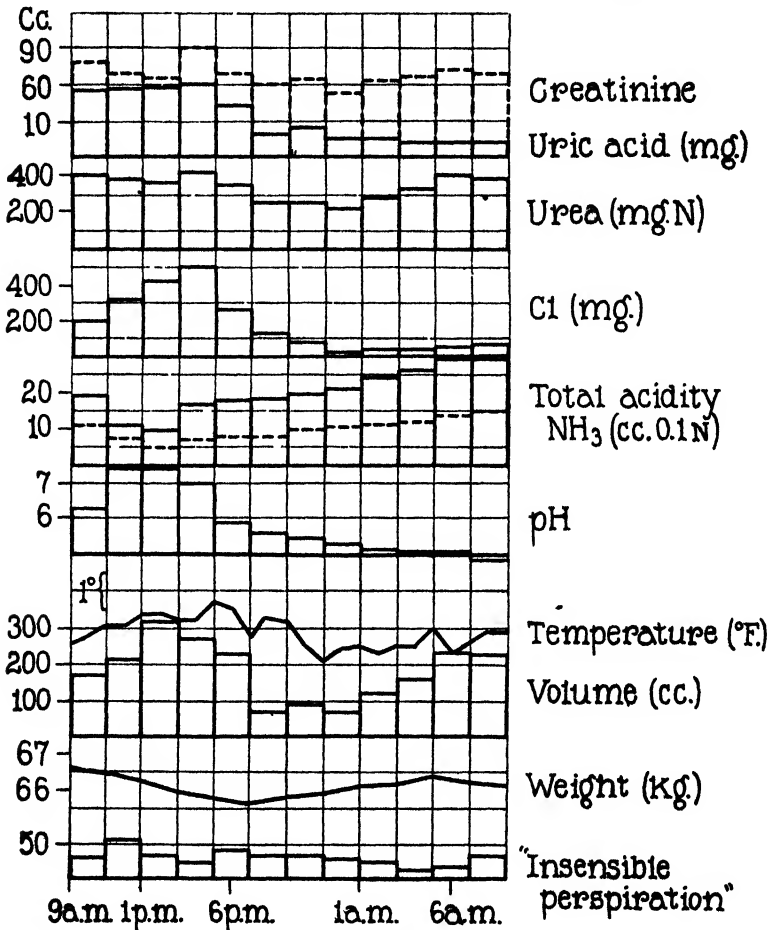


CHART 2. 200 cc. of H_2O hourly, at room temperature. Room temperature 64-68°. Humidity 37 to 61 per cent. Subject G.W.C. June 18, 1923.

increased protein catabolism. In the 200 cc. experiments, body temperature did not follow volume nearly so closely as in the 100 cc. experiments (Chart 2), although there is a general

association.⁴ In these experiments humidity was not as constant as in the experiments carried on earlier in the spring; also the water was given at room temperature rather than near body temperature, because it had been shown that this did not affect the 100 cc. experiments. With the larger amount of water, it does not follow that this is so, and closer parallelism may have been obtained if the water had been given at body temperature. However, in some 200 cc. experiments (with glucose) to be reported below, the water was given at 38–40°, and the parallelism was no closer.

*Creatinine Excretion (100 and 200 Cc. Experiments)—
Table II.*

The question of the regularity of creatinine excretion over short intervals has recently been reopened by Schulz (9) and Neuwirth (1). Schulz, who collected 2 hour samples during the day, attributes the regularity of Shaffer's figures to the longer collection periods during which minor variations counter-balanced. Neuwirth, who collected hourly samples, takes his data to indicate that creatinine excretion is not regular during brief fasts.

I believe that Neuwirth has overemphasized the irregularities in his data, that creatinine excretion *per se* is quite regular during short periods without food, and that the minor variations are due to variations in the urine volumes when these are relatively small. Table I shows the relation between creatinine excretion and water excretion, and also shows that the smaller excretion of creatinine usually found during the night is not due to sleep, but rather to water retention during this period. This is especially seen in Experiment 3, in which the subject had a long afternoon nap.

Some experiments are detailed in Table II, which also shows volumes.

⁴ The variations in temperature are slight, and possibly better experimental conditions may be necessary to make them apparent. It is a fault in these experiments that sitting up in bed during the day undoubtedly facilitates dispersion of heat. It is hoped to improve future experiments in this respect by using a sleeping bag.

	Experiment 1. W.H.J.A.			Experiment 2. G.E.S.-1.			Experiment 3. B.P.W.			Experiment 4. G.W.C.			Experiment 5. G.E.S.-2.			Experiment 6. McI.		
Time.	Urine volume.	Creatinine.		Urine volume.	Creatinine.		Urine volume.	Creatinine.		Urine volume.	Creatinine.		Urine volume.	Creatinine.		Urine volume.	Creatinine.	
a.m.	cc.	mg.		cc.	mg.		cc.	mg.		cc.	mg.		cc.	mg.		cc.	mg.	
7																		
8	12*	49					88*	65					307†	87				
9				160	60													
10	24	95					136	70					347	72				
11				184	69					175†	78					65*	29	
12	25	84					250‡	83					230	64				
p.m.																		
1				139	63					210	70					245	52	
2	84	86					245‡	91					355	73				
3				84	78					322	66					200	55	
4	125	98					127	68					325	62				
5				98	68					270§	90					192	55	
6	100	91					93	76					160	66				
7				27	73					232	68					247	49	
8	80	80					42	75					185	61				
9				17(a)¶	63					70	61					162	53	
10	85	95					58	66					130	69				
11				21	71					89	63					150	55	
12	25 ¶	82					55	76					67 ¶	56				
a.m.																		
1				28	72					68	54					185	53	
2	35	84					34	71					180	66				
3				20(b) ¶	63					122	63					147	51	
4	27 ¶	78					156	74					242	65				
5				38	80					160	67					172	51	
6	27	71					176	75					212	71				
7				110	65					227	73					135	53	
8													235	69				
9										217	70					235	50	
11																227	55	
p.m.																		
1																172	55	

Comments on Table II.⁵

* If volume remains low during the first morning collection, creatinine elimination remains low, with an increase to a higher level when urine volume increases (Experiments 1, 3 (?), and 6).

† If volume is high for the first collection, a high creatinine may be shown, with a later decrease to a lower level (Experiments 4 and 5).

‡ A sharp drop in urine volume may be accompanied by a sharp decrease in creatinine excretion, more especially during the night retention period. This might be attributed to imperfect collection when it is followed by a compensatory high excretion (Experiment 2 (b)), but this cannot be a factor in Experiments 1, 2 (a), and 5.

§ In Experiment 3, the high figures may be a result of previous retention (especially during the night). The average creatinine for 4 hours previous = 67 mg.; for the 16 hours after, 72 mg.

§ This anomalous result, together with the single instance in Neuwirth's data (Experiment 5, 5 to 6 p.m.) which cannot be accounted for by the above considerations, are not sufficient, in the writer's opinion, to indicate any real irregularity of creatinine production.

It will be noticed in Experiment 6, where water excretion is more regular than in any other experiment, creatinine variations are exceedingly slight. The regularity of Shaffer's results is to be attributed to the fact that in his experiments the minimal water excretion per hour, more particularly during the period when retention of water, and consequently of creatinine, was most likely to occur, was higher than in Neuwirth's experiments or in mine.⁶

Chlorides.—Chart 2 shows the results in a typical experiment. Our results do not represent anything new and will not be detailed. In one of the experiments which showed smaller variations in volume than were found in any other experiments, chloride excretion was lowest in amount (maximum 176 mg., minimum 59 mg. of Cl per hour) and showed least variations.

Uric Acid.—In three of the four 100 cc. experiments low uric acid was found in the morning after waking even though water excretion was at or near its maximum. This has been seen, but not explained, by Mendel and Stehle (10). It is to be noted that this was not met with in the three 200 cc. experiments—

⁵ This analysis may be applied to Neuwirth's data as well.

⁶ In view of the results here reported, it is difficult to see how creatinine excretion is related to muscle tonus.

Chart 2 is typical. In general the results confirm those of others (4.14).

The results with chlorides, urea, and uric acid in the 100 and 200 cc. experiments may be summarized as follows: When the diminution of volume⁷ ushers in the period of water retention, there is a sharp decrease in the excretion of these substances. Minimum Cl excretion during the night may be less than one-tenth the maximum during the day; uric acid may decrease to as little as one-fourth its maximum—the well known washing out effect. Chlorides and uric acid increase slightly on the 2nd morning when water excretion increases, but they do not approach their pristine level. Urea, on the other hand, increases on the 2nd morning and may attain values higher than on the 1st day, doubtless a result of increased protein catabolism.

Hydrogen Ion Concentration, Titratable Acidity, and Ammonia.

With hydrogen ion concentration,⁸ titratable acidity, and ammonia, the case is quite different. Hydrogen ion concentration shows a marked increase during the period of water retention. The results of two experiments with 200 cc. of water are shown in Table III. During the period when the volume is

⁷ Volumes in 200 cc. experiments are recorded in Table III.

⁸ The paper of Marshall (11) had not come to the writer's attention when pH determinations were begun. In the experiments with glucose the urines were collected as Marshall recommends. In the 1st day's samples (until 4 p.m.) and the first two samples on the morning of the 2nd day in these glucose experiments pH was determined on: (a) 2 cc. of urine diluted to 10 cc.; (b) 10 cc. of undiluted urine; (c) on 2 cc. diluted to 10 cc., but allowing the urine to fall a considerable distance through the air into the test-tube; and (d) on a sample of urine taken some time after it had been standing in a flask under toluene, after having been poured into this flask from the receiving cylinder. The results did not differ materially from one another. (The maximum difference was 0.1 pH, except once, when it was 0.2 pH.) This shows that it is permissible to allow the night urines to stand until morning. It also shows that the results of the first two experiments are essentially correct, even though loss of CO₂ was not guarded against in collecting the samples. Indeed, since Marshall showed that failure to use his precautions would give pH results lower than the truth in alkaline urines especially, it would be expected that if any errors in our determinations had been made, the correct result would show higher pH than the actual value in the earlier (more alkaline) urine and that the range in pH obtained in our experiments would be even greater than the data indicate.

decreasing from the maximum of 322 cc. to about one-fourth of this (70 cc.) G.W.C. shows a tenfold increase in hydrogen ion concentration. At the very beginning of decreased water elimination titratable acidity shows a large increase, and there is a concomitant rise in ammonia. G. E. S.-2 shows a marked increase in hydrogen ion concentration with the halving of volume, and this increase is again preceded by increased titratable acidity and, to a less extent, ammonia. (In two 100 cc. experiments also, the ammonia changes were marked.)

Experiments with 200 Cc. of Water plus 10 Gm. of Glucose.

Part of the data from these experiments are shown in Table III, Experiments 8 and 9. Fasting might reasonably be regarded as the cause of the increase in hydrogen ion concentration, acidity, and ammonia noted above. Therefore, 240 gm. of glucose, sufficient to cover one-half to one-third the energy requirements and which should prevent even a minor depletion of the carbohydrate reserve, was administered in twenty-four doses of 10 gm. each. The maximum body temperature now shifts to the late afternoon or evening.⁹ The increase in hydrogen ion concentration is not now so great as in the fasting experiments, but is marked, nevertheless. A sharp increase still accompanies the sharp decrease in water excretion. The titratable acidity in Experiment 9 increases with hydrogen ion concentration, while ammonia shows a small change in the same direction.¹⁰

There is, then, an inverse relation between hydrogen ion concentration, titratable acidity, and ammonia, on the one hand, and water excretion by the kidneys on the other. But this does not hold beyond the period of water retention; it does not hold when water excretion increases on the 2nd morning. Hydrogen ion concentration does not now decrease, but shows rather a tendency in the opposite direction, and this is so whether glucose is given or not.

⁹ That, during fasting, the diurnal temperature shows a tendency to a maximum in the morning rather than in the afternoon was noted by Benedict (8).

¹⁰ Unfortunately one series of experiments had to be discarded. It is necessary to check up not only the neutrality of each bottle of potassium oxalate, but samples from different parts of the same bottle. The top part of a fresh bottle contained neutral oxalate, the bottom was quite acid.

DISCUSSION.

On the question of the relation between volume and acidity or ammonia of urine, evidence was first obtained by Henderson and Palmer (12) who, on arranging 122 24 hour urines into four groups according to pH, noted that average volume increased with average pH. The literature on the subject is on the whole confusing. Quite recently, Hubbard and Munford (13), who discuss some of this literature from statistical treatment of the 147 2 hour urine samples collected by them, discovered an inverse relation between volume on the one hand and hydrogen ion concentration, titratable acidity, and ammonia, on the other. In the three single experiments detailed by them, no relation is to be seen, however. They also found that when hydrogen ion concentration was relatively constant, ammonia and volume vary not inversely, but directly—a condition which may be noted in our experiments on the 2nd morning.

In the period over which the present observations extend, it is of interest to note that the changes in hydrogen ion concentration occur during the time when volume changes occur, and that there is no inverse proportion except in this period. Extension of the time of observation into the 2nd day would be of especial interest, since it is not to be expected that so great variations in pH would occur during this second 24 hour period. It may be that qualitative changes in acid excretion determine the changes in water excretion, or, possibly, *variation* in C_H rather than C_H itself, is related to changes in urine volume. Changes in hydrogen ion concentration of the urine may be an expression of changes in the hydrogen ion concentration of the body tissues, which may be a potent factor in determining water excretion. For instance, as the tissues become acid, water may be imbibed. This would occur over a certain range of hydrogen ion concentration and would not necessarily occur for any other range. On this basis water excretion should be more nearly level on the 2nd day of such an experiment.

Results thus far do not warrant any discussion of such a theory, although it is the intention to look into it.

There are various results in the literature which indicate inhibition of water on acid diet and the reverse on alkaline diet

(14), and also showing that when the alkalinity of the tissues is increased, urine volume increases. Collip's (15) data, for instance, show that increased volume and increased alkalinity of the urine follow overbreathing.

Any means by which the night retention period could be eliminated should provide knowledge regarding the regulation of water excretion. It is hoped to find out if it can be eliminated by the administration of regular amounts of sodium bicarbonate, for instance. But it will be possible to plan such an experiment more intelligently after the excretion of inorganic salts and of organic acids under the conditions of the above experiments has been determined, and it is known what are the particularities in the changes in hydrogen ion concentration, etc. Such a study is now under way. It is not the present intention to proceed further with the work on creatinine, uric acid, and urea.

SUMMARY.

A study of 2 hour urines during 24 hours or more shows:

1. When 100 or 200 cc. of water are given hourly, there is a relation between urine volume and body temperature.

2. Whether 100 cc. of water, 200 cc. of water, or 200 cc. of water plus 10 gm. of glucose are given hourly, there is a negative water balance during part of the day, and a retention of water at night. Associated with the development of this retention period is a large increase in the hydrogen ion concentration and also in titratable acidity and ammonia of the urine.

3. The retention period ends about 7 a.m. on the 2nd morning. At this time hydrogen ion concentration does not alter appreciably, and titratable acidity shows no marked changes, while ammonia excretion increases.

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THE SPECIFIC ROTATIONS OF HEXONIC AND 2-AMINO- HEXONIC ACIDS AND OF THEIR SODIUM SALTS.

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In connection with work on the configuration of amino sugars it was desirable to find the direction of rotation of the carbon atom 2 in monocarboxylic sugar acids and in aminohexonic acids. In the series of sugar acids it was concluded that when the hydroxyl of the carbon atom 2 was in the same position as in gluconic acid, the rotation was to the right and *vice versa*. This conclusion was reached by Levene¹ and Levene and Meyer² on the basis of existing data on the rotation of the metallic and alkaloidal salts of sugar acids and on their phenylhydrazides. Hudson has later corroborated the conclusions of Levene and Levene and Meyer and has further pointed out that, in many instances, the direction of the rotation of an acid was determined by the rotation of carbon atom 2. Hudson³ made his observations on phenylhydrazides and Hudson and Komatsu,⁴ on the amides. The conclusions of both Levene and of Hudson were substantiated by the work of Weerman.⁵ However, it had been known, and it was recently emphasized by Hudson and Komatsu⁴ that in the case of many 2-hydroxy acids the rotations of the free acids are in the opposite direction from the respective metallic salts, phenylhydrazides, and amides. The series of hexonic acids, however, is not completely investigated in this respect. Observations are limited to *d*-galactonic acid which is known to rotate to the left whereas its

¹ Levene, P. A., *J. Biol. Chem.*, 1915, xxiii, 145.

² Levene, P. A., and Meyer, G. M., *J. Biol. Chem.*, 1916, xxvi, 355; 1917, xxxi, 623.

³ Hudson, C. S., *J. Am. Chem. Soc.*, 1917, xxxix, 462; 1918, xl, 813.

⁴ Hudson, C. S., and Komatsu, S., *J. Am. Chem. Soc.*, 1919, xli, 1141.

⁵ Weerman, Dissertation, Amsterdam, 1916.

salts, phenylhydrazides, and amides rotate to the right. Gluconic acid soon after solution is known to show doubtful levo-rotation or to be inactive and its lactone formation is accompanied by change of rotation. The salts and other derivatives of this acid are dextro-rotatory. In view of this lack of information the direction of rotation of the other hexonic acid was determined. It was found that mannonic, idonic, and altronic acids rotated to the right, whereas allonic and gulonic acids rotated to the left. This information together with that of the already known rotations of gluconic and galactonic acids permit the formulation of a general rule to the effect that, *in the series of hexonic acids, the free acids have a rotation in the direction opposite from that of the salts or derivatives of the same acids.*

In the series of the 2-aminohexonic acids the conditions are different. The free acids show a comparatively small rotation and, in the past, for the sake of greater accuracy, they were measured in hydrochloric acid solution.⁶ Measurements were now made in a solution of sodium hydroxide. It was found that in the arabinohexosaminic acids and in the xylohexosaminic acids the rotation of the carbon atom 2 was in the opposite direction from the one previously found. On the other hand, in *d*-lyxohexosaminic and *d*-ribosehexosaminic acids the rotation of carbon atom 2 remained the same in both alkaline and acid solution.

Other peculiarities were also noted in the case of the sodium salts of aminohexonic acids. In the case of arabinohexosaminic and lyxohexosaminic acids the epimeric acids rotate in the same direction. That means that in the case of these four salts the direction of the rotation of carbon atom 2 does not determine the direction of rotation of the salt. Furthermore, the numerical values of the rotations of carbon atom 2 of the aminohexonic acids show greater variation in alkaline than in acid solution.

The present observations may be of great importance in the discussion of the configuration of 2-aminohexoses and particularly that of chitosamine. This discussion will be presented in a separate communication.

⁶Levene, P. A., Hexosamines, their derivatives, and mucins and mucoids, Monograph of The Rockefeller Institute for Medical Research, No. 18, New York, 1922.

EXPERIMENTAL PART.

The hexonic acids in our possession were either in the form of the calcium salt, or in the form of their lactones. The calcium salt was rapidly dissolved in water containing a slight excess of hydrochloric acid, previously chilled to 0°C. When the lactone was present it was allowed to stand overnight with a slight excess of alkali. The solution was then chilled to 0°C. and this solution was acidulated with an aqueous solution of hydrochloric acid chilled to 0°C.

Gluconic Acid.—The calcium salt was used. The rotation was as follows:

$$[\alpha]_D^0 = 0.0^\circ$$

Mannonic Acid.—The lactone was used.

$$[\alpha]_D^0 = \frac{+0.78^\circ \times 100}{2.5 \times 2} = +15.6^\circ$$

Idonic Acid.—Only the brucine salt of this acid was on hand. This was converted into the barium salt which was used for the measurement. The quantity on hand was too small to determine the rotation quantitatively. The direction, however, was to the right. In view of the fact that the lactone is levo-rotatory, there seems to be no doubt that idonic acid rotates to the right.

Gulonic Acid.—The lactone was used. The specific rotation was as follows:

$$[\alpha]_D^0 = \frac{+0.08^\circ \times 100}{2.5 \times 2} = +1.6^\circ$$

Since the rotation of the lactone is strongly levo-rotatory and the observed rotation of the gulonic acid only slightly levo-rotatory it is possible that the rotation of the pure acid is slightly dextro-rotatory.

Galactonic Acid.—The lactone was used. The rotation was

$$[\alpha]_D^0 = \frac{-0.40^\circ \times 100}{2.5 \times 2} = -8.0^\circ$$

Talonic Acid.—Too little of the brucine salt was on hand to permit its conversion into the free acid.

Allonic Acid.—The lactone was used. The rotation was as follows:

$$[\alpha]_D^{20} = \frac{-0.50^\circ \times 100}{2.5 \times 2} = -10.0^\circ$$

Altronic Acid.—The calcium salt was used.

$$[\alpha]_D^{20} = \frac{+0.40^\circ \times 100}{2.5 \times 2} = +8.0^\circ$$

TABLE I.

Showing the Specific Rotations of the Free Acids and of Their Derivatives (All of the d-Series).

	Free acids.* [α] _D ²⁰	Na salts † [α] _D ²⁰	Phenylhy- drasides.‡ [α] _D ²⁰	Amides.§ [α]
Gluconic.....	0.0	+11.78	+18.0	+31.2
Mannonic.....	+15.6	-8.82	-10.5	-17.3
Idonic.....	+	-2.52	-15.1	.
Gulonic.....	-1.6	+12.68	+13.45	+15.2
Galactonic.....	-8.0	+0.40	+12.2	+30.0
Talonic.....	?	?	+4.35	
Allonic.....	-10.0	+4.30	+25.88	
Altronic.....	+8.0	+4.05	-15.8	

* Recent observation.

† Observations by Levene and Meyer (Levene, P. A., and Meyer, G. M., *J. Biol. Chem.*, 1916, xxvi, 365).

‡ Observations by Levene and Meyer (Levene, P. A., and Meyer G. M., *J. Biol. Chem.*, 1917, xxxi, 625).

§ Observations by Hudson and Komatsu (Hudson, C. S., and Komatsu, S., *J. Am. Chem. Soc.*, 1919, xvi, 1142).

TABLE II.

Acids.	In 5 per cent NaOH [α] _D ²⁰	In 2.5 per cent HCl. [α] _D ²⁰
Chitosaminic.....	-1.3° c = 5.0.	-15°
Epichitosaminic.....	-5.0° " = 5.0.	+10.0°
Dextro-d-xylo-2-aminohexonic.....	-16° " = 2.5.	+14.0°
Levo-d-xylo-2-aminohexonic.....	+2.0° " = 2.5.	-11.0°
Chondrosaminic.....	-15° " = 2.5.	-17.0°
Epichondrosaminic.....	-1.8° " = 2.5.	+8.0°
Dextro-d-ribo-2-aminohexonic.....	+2.0° " = 2.5.	+12.5°
Levo-d-ribo-2-aminohexonic.....	-15.0° " = 2.5.	-26.0°

2-Aminohexonic Acids.—The specific rotation of all these acids was measured in a uniform way. A 2.5 per cent solution of the acid in a 5 per cent solution of sodium hydroxide cooled to 0°C. was freshly prepared and measured immediately after the solution was completed. Chitosaminic and epichitosaminic acids were measured in 5 per cent concentrations.

The specific rotations are compared with the results of previous measurements in a solution of hydrochloric acid in Table II.

PREPARATION OF α -MANNOSE.

SECOND PAPER.

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(Received for publication, December 17, 1923.)

The preparation of a mannose with a specific rotation of $+30^\circ$ in water and $+35^\circ$ in 80 per cent alcohol was reported in a previous paper. On theoretical considerations, Hudson and Yanovski arrived at the latter value for the specific rotation of α -mannose. The behavior of the new form of mannose, as was stated in the earlier paper, agreed with the assumption of its being the α form. The substance, however, had a few peculiarities inconsistent with this theory. The most striking contradiction was presented by the value for k_1 and k_2 derived from the measurement of the mutarotation of the α form, which was higher than the value for the $k_1 + k_2$ obtained from observations on the mutarotation of the β form. It was, however, realized that the measurements required repetition under more favorable conditions; namely, in aqueous solution at low temperatures. These measurements were now carried out at 1.5°C . Under these conditions, it was found that the value for $k_1 + k_2$, obtained from observations on the mutarotation of the α form, was identical (within the limits of experimental errors) with that obtained from observations on the β form.

The measurements were then repeated at 25°C . in 80 per cent alcoholic solution, that is, under the conditions of the earlier experiments and here, too, it was found that the value of $k_1 + k_2$ was the same regardless of the form on which the observations were made.¹ The discrepancy of the last year's results was

¹ The α form employed in these experiments was prepared by crystallization from glacial acetic acid. It seems that acetic acid is more readily removed than pyridine. The details of preparation are given in the experimental part.

undoubtedly due to traces of pyridine which adhered to the α form, notwithstanding the fact that it was purified by repeated extractions with 80 per cent alcohol.

Furthermore, from the observation of the rate of solubility of the α form k_1 was calculated and from that of the β form k_2 was calculated and the sum of the $k_1 + k_2$, each derived independently, was practically identical with the sum $k_1 + k_2$ obtained from measurements of the mutarotation. Thus, the phenomena connected with the mutarotations of the old and of the new forms of mannose contain nothing contradictory to the assumption that the new form is the α form of mannose. There remains, however, some peculiarities in the equilibria between the two forms of mannose which are in need of further explanation.²

EXPERIMENTAL PART.

Preparation of α -Mannose.—All the methods for preparation of α -mannose described in the previous publication continue to yield good results. The most rapid and convenient method, however, is as follows:

² The previous communication (Levene, P. A., *J. Biol. Chem.*, 1923, lvii, 329) contains three errors. Hudson and Dale's directions for the preparation of β -glucose were erroneously quoted. These authors prepared β -glucose by crystallizing it not from cold, but from hot, glacial acetic acid. This error, however, does not affect the general argument, as under the same conditions, mannose crystallizes in the α form. In this connection a peculiar occurrence must be mentioned. Many workers have tried to prepare α -mannose and all have failed, but since the α form was once crystallized in our laboratory, it has become impossible in our laboratory to recrystallize the β form without converting it into the α form. Mannose prepared in the usual way still crystallizes in the β form, but on recrystallization, it is always converted into the α isomer. On two occasions the β form was recrystallized without change, but when the recrystallization was repeated under the same conditions, again the α form crystallized. We are still at work on conditions which would permit the recrystallization of the β form. At present the pure β isomer is prepared by repeated extraction of the crude material with 80 per cent alcohol.

Another error was overlooked in the first paper; namely, in the expression $(k_1 + k_2) = \frac{1}{t} \log \frac{r_0 - r_\infty}{r_t - r_\infty}$, the factor $\frac{1}{t}$ was omitted.

The third error was contained in the table on page 335. The equilibrium rotation of the β form is $+1.34^\circ$ and not $+0.34^\circ$.

A solution of mannose is made in the proportion of 100 gm. of mannose to 25 cc. of water. This solution is chilled to 0°C. and to the solution 400 cc. of chilled glacial acetic acid are added. After 4 to 5 hours of standing, the α form can be filtered off. The crude product has a specific rotation of +28 to +29°. In order to remove all adhering acetic acid and the small portion of the β form the crude material is extracted eight times with 80 per cent (by weight) alcohol. The extraction is carried out at 20°C. and 200 cc. of the alcohol are used for each 100 gm. of the sugar.

Mutarotation of α -Mannose in Aqueous Solution.

Time.

 $[\alpha]_D^{25}$

$$k_1 + k_2 = \frac{1}{t} \log \frac{r_0 - r_\infty}{r_t - r_\infty}$$

10 per cent aqueous solution. A 200 mm. tube was used for measurement.

min.		
0	+5.62	
19	+5.30	0.0029
40	+5.00	0.0028
64	+4.67	0.0029
93	+4.37	0.0029
117	+4.08	0.0031
Equilibrium.....	+2.95	Average...0.0029

20 per cent aqueous solution. A 100 mm. tube was used.

0	+5.82	
20	+5.50	0.0027
42	+5.11	0.0029
62	+4.77	0.0032
86	+4.43	0.0032
Equilibrium.....	+2.95	Average...0.0030

34 per cent aqueous solution. A 100 mm. tube was used.

0	+9.70	
23	+9.02	0.0021
47	+8.35	0.0025
78	+7.62	0.0027
103	+7.13	0.0030
125	+6.80	0.0030
145	+6.47	0.0031
164	+6.28	0.0031
190	+6.03	0.0031
Equilibrium.....	+4.80	Average...0.0028

Mutarotation of α -Mannose in 80 Per Cent Alcoholic Solution.

8.0 gm. of mannose were shaken in 30 cc. of 80 per cent alcohol for 4 minutes and filtered. A 200 mm. tube was used.

Time.	$[\alpha]_D^{25}$	$k_1 + k_2 = \frac{1}{t} \log \frac{r_0 - r_\infty}{r_t - r_\infty}$
<i>min.</i>		
0	+5.95	
22	+5.53	0.00580
42	+5.24	0.00523
72	+4.88	0.00550
91	+4.76	0.00520
113	+4.62	0.00520
Equilibrium.....	+4.16	Average...0.00540

Mutarotation of β -Mannose.—The rate of mutarotation of β -mannose in aqueous solution at 1°C. was measured by Hudson and Sawyer and was repeated here in order to have data on the α and β forms established under exactly the same conditions and also to test the accuracy of our measurements. The results are as follows: Time is given in minutes and decimal logarithms are used for calculations.

10 per cent of β -mannose solution. A 200 mm. tube was used for measurement.

Time.	$[\alpha]_D^{15}$	$k_1 + k_2 = \frac{1}{t} \log \frac{r_0 - r_\infty}{r_t - r_\infty}$
<i>min.</i>		
0	-2.93	
19	-2.25	0.0029
45	-1.54	0.0030
65	-1.05	0.0029
88	-0.48	0.0029
Equilibrium.....	+2.78	Average...0.0029

Hudson and Sawyer found at 1°C., $k_1 + k_2 = 0.0030$.

Mutarotation in 80 Per Cent Alcohol.

3.0 gm. of β -mannose were dissolved in 30.0 cc. of 80 per cent alcohol. A 200 mm. tube was used.

Time.	$[\alpha]_D^{25}$	$k_1 + k_2 = \frac{1}{t} \log \frac{r_0 - r_\infty}{r_t - r_\infty}$
min.		
0	-0.67	
21	-0.25	0.00542
51	+0.26	0.00566
101	+0.70	0.00601
145	+0.90	0.00594
Equilibrium.....	+1.15	Average...0.00575

Maximum Rate of Solution of α - and β -Mannoses in 80 Per Cent Alcohol at 15°C.—Hudson and Lowry have shown that the rate of conversion of a unit concentration of one isomer into the other can be estimated by measurement of the rate at which a given sugar reaches its maximum solubility. The value of k , the coefficient of this conversion, is given by the expression $\frac{1}{t} \log \frac{S_0 - S_\infty}{S_t - S_\infty}$

α -Mannose.—25.0 gm. of α -mannose, dried under reduced pressure at 55°C., were suspended in 125 cc. of 80 per cent alcohol in a flask provided with a ground stopper. The flask was continually shaken in a water bath maintained at 15°C. The value S_0 was estimated on the basis of the rotation of a sample taken after an interval of 5 minutes. 2 drops of ammonia were added and the sample was allowed to stand. S_∞ was measured in the following way. After the sugar remained in contact with the alcohol for 24 hours, a few drops of ammonia were added and the mixture was allowed to stand an additional hour. To avoid any marked change in concentration of the alcohol during the long interval, the flask was covered with paraffin. The measurements were taken in a 100 mm. tube.

Time.	$[\alpha]_D^{15}$	$k_1 = \frac{1}{t} \log \frac{S_0 - S_\infty}{S_t - S_\infty}$
min.		
0	+5.40	
39	+5.60	0.00170
87	+5.86	0.00190
127	+6.04	0.00204
169	+6.20	0.00210
∞	+6.82	Average...0.00193

β -Mannose.—10.0 gm. of β -mannose, dried at reduced pressure at 55°C., were suspended in 125 cc. of 80 per cent alcohol. All the details of the experiment were the same as with the α form. The measurements were taken in a 200 mm. tube.

Time	$[\alpha]_D^{15}$	$k_2 = \frac{1}{t} \log \frac{S_0 - S_\infty}{S_t - S_\infty}$
min.		
0	+1.05	
37	+1.15	0.00030
81	+1.34	0.00042
124	+1.49	0.00043
169	+1.70	0.00048
∞	+4.86	Average...0.00041

From these data there is noticed a consistent small deviation from the monomolecular course of the reaction. It is not possible at present to state whether the changes are due to the limits of error of the method.

THE OPTICAL BEHAVIOR OF 2,5-ANHYDROGLUCOSE, OF 2,5-ANHYDROGLUCONIC ACID, AND OF 2,5-ANHYDROMANNONIC ACID.

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The mutarotation of sugars has been explained by the assumption of an oxidic structure between carbon atom 1 and of one of the other carbon atoms of the sugar molecule. In recent years it was further assumed that in aqueous solution, the aldehydic and the oxidic forms exist in dynamic equilibrium, the oxidic form predominating, particularly at the time when the equilibrium between the α and the β forms is established. As yet, however, the aldehydic form of a sugar has not been isolated either in crystalline condition or in solution. The properties and behavior of the aldehydic form have never been studied although on general principles it has been assumed to be the more reactive form and probably the form which undergoes oxidation in the living organism. It was, therefore, of interest to possess a sugar which *a priori* could have only the aldehydic structure. Such a sugar is 2,5-anhydroglucose.¹ It will be seen from the photographs of the model (Figs. 1 and 2) that carbon atoms 1 and 4 are situated in transpositions with respect to the plane formed by the ring between carbon atoms 2 and 5 and that a second ring between carbon atoms 1 and 4 can no longer be established. Again, the positions of carbon atoms 1 and 3 are divergent (Fig. 2) and hence these two atoms cannot readily form a ring. In accordance with these considerations, 2,5-anhydroglucose does not manifest the phenomenon of mutarotation. As compared with other sugars, it shows greater power to reduce a solution of potassium permanganate. The study of the details of the reactivity of the sugar has to be post-

¹ Levene, P. A., *J. Biol. Chem.*, 1919, xxxix, 69.

poned until sufficient material has been prepared. Unfortunately, the preparation of the sugar is a very laborious and costly process.

Similarly to the sugars, the monocarboxylic sugar acids also manifest the phenomenon of mutarotation. In the case of the acids the mutarotation is due to lactone formation. The reproductions of the models (Figs. 1 to 3) clearly show that no lactone formation can take place in 2,5-anhydrogluconic and 2,5-anhydromannonic acids, and in truth these two acids retain a constant

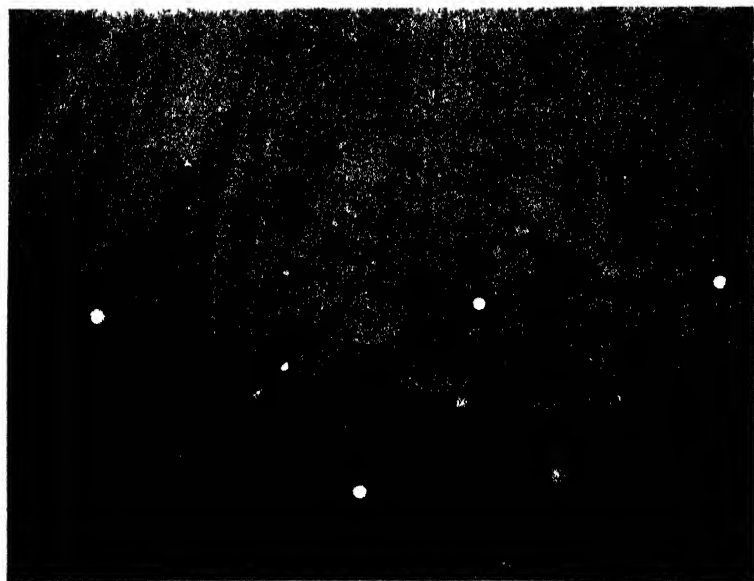


FIG. 1. 2,5-Anhydroglucose.

rotation in aqueous solution. The optical behavior of these two acids differ from other hexonic acids in still another respect. In the ordinary sugar acids the rotation of the carbon atom 2 and often of the acids themselves is in the opposite direction from that of their salts. In the case of the 2,5-anhydrosugar acids the specific and the molecular rotations are practically the same in direction and in magnitude, both in the free acids and in their salts.

In the case when an acid and its salts had distinctly different rotations either in direction or in magnitude, the tendency was to

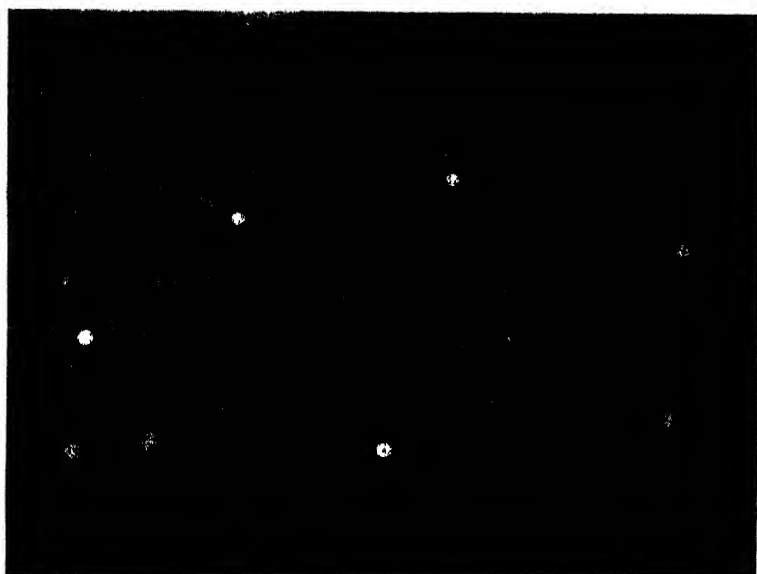


FIG. 2. 2,5-Anhydroglucose.

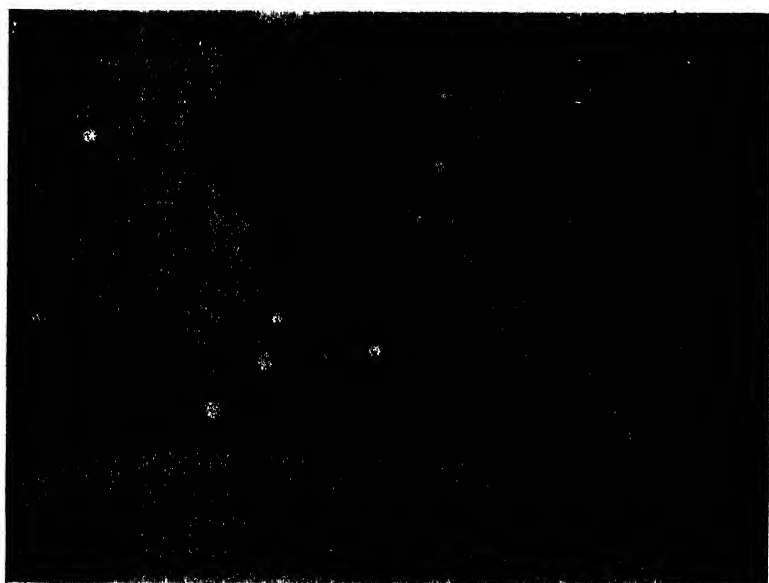


FIG. 3.

138 Optical Behavior of 2,5-Anhydroglucose

ascribe the difference to the variations in the degree of electrolytic dissociation of the free acid and of its salts. The literature on this subject is discussed in Landolt's "Das optische Drehungsvermögen."² In the case of the free sugar acids and their salts the variations in the optical behavior cannot be explained by the different degrees of electrolytic dissociation since the salts, amides, and phenylhydrazides rotate in the same direction, whereas the free acids rotate in the opposite direction. Thus, the most highly dissociable derivatives and those which do not dissociate at all rotate in one direction, whereas the moderately dissociable substance rotates in the opposite direction. The more plausible explanation of the difference of rotation in the salts and in the free acids lies in the assumption of a different orientation of the carbon atoms within the molecules of the free acid and of its salt. Hence, when the opportunities for reorientation of the atoms are restricted as in 2,5-anhydro acids, then the rotation remains identical in the acid and in its salt. Landolt has already suggested a similar explanation for the change in the optical rotation of sugar solutions depending on concentration or on the character of the solvent.

EXPERIMENTAL PART.

Oxidation of Epichitose by Means of Potassium Permanganate.—Equal quantities of α -glucose, α -mannose, α -galactose, and 2,5-anhydroglucose were dissolved in 1 cc. of water and an equal volume of 0.0025 N potassium permanganate was added to each test-tube. The tube containing 2,5-anhydroglucose was decolorized in 10 minutes. The next in order was mannose, which was decolorized in 4 hours.

Chitonic Acid.—The calcium salt was dissolved in a 5 per cent solution of hydrochloric acid, chilled to 0°C., and the rotation measured immediately.

Initial.	After 24 hours.
$[\alpha]_D^0 = \frac{+1.15^\circ \times 100}{3 \times 1} = +38.3^\circ$	$[\alpha]_D^{17} = \frac{+1.15^\circ \times 100}{3 \times 1} = +38.3^\circ$

² Landolt, H., *Das optische Drehungsvermögen organischer Substanzen und dessen praktische Anwendungen*, Brunswick, 2nd edition, 1898.

Chitic Acid.—Pure crystalline chitic acid was used. It was dissolved in water chilled to 0°C.

Initial.

$$[\alpha]_D^{20} = \frac{+1.90^\circ \times 100}{3 \times 1} = +63^\circ$$

After 24 hours.

$$[\alpha]_D^{20} = \frac{1.86^\circ \times 100}{3 \times 1} = +62^\circ$$

Specific Rotation of the Free Acids and Their Calcium Salts.

	Free acids.	Ca salts.
Chitonic.....	+33.65°	+30.3°
Chitic.....	+70.29°	+63.0°

THE PENTACETATE OF α -MANNOSE.

By P. A. LEVENE.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, December 29, 1923.)

Two forms of mannose have now been prepared, the old form with a specific rotation of -15° and a new form with a specific rotation of $+30^\circ$ in water and $+35^\circ$ in 80 per cent alcohol. Both have the same equilibrium rotation of $+18^\circ$. The common form was always regarded as the β isomer of the buteleneoxidic form of mannose. On the basis of the rate of maximum solubility of this form Hudson and Yanovski calculated the specific rotation of the α form to have the rotation of the recently discovered new form of mannose; namely, $+35^\circ$ in 80 per cent alcohol. Thus the difference of the molecular rotations of these two forms is 9,180. In this respect the pair of mannoses are an exception to a rule derived by Hudson; namely, that the difference of the molecular rotations of a pair of α - and β -aldoses has a value in the neighborhood of 17,000.

On the other hand, the pentacetate of β -mannose with a molecular rotation of $-9,800$ was converted by Hudson and Dale into an isomer with a molecular rotation of 21,400, thus giving a difference of 31,200, which approaches the difference of the pentacetates of the α - and β -galactoses. The second pentacetate of mannose was regarded as the α isomer.

Since the difference of the molecular rotations of the two mannoses is irregular and that of the two pentacetates practically normal and since the second pentacetate of mannose was not prepared directly from α -mannose, it was uncertain that it was a derivative of the newly discovered form of mannose with a specific rotation of $+30^\circ\text{C}$. It was therefore necessary to convert the new form of mannose into the pentacetate. Experience has shown that the structure of a sugar remains the least changed when acetylation is carried out in pyridine solution at 0°C . In the

case of α -mannose, the acetylation proceeded quite normally. The product however crystallized very slowly. When allowed to stand very long, the original sticky oil turned into a solid mass imbedded with crystals. It was not possible to obtain an accurate estimate of the yield of the crystalline pentacetate for the reason that too much of the oil adhered to the crude material and too much of the crystalline product was lost in the process of purification. The weight of the twice purified substance was equal to 40 per cent of that of the original mannose. The specific rotation of the purified material, recrystallized either out of ether or out of water, had a specific rotation of $+ 57.6^\circ$. Thus, the pentacetate prepared directly from the α -mannose is identical with the pentacetate prepared by Hudson and Dale by the catalytic action of zinc chloride on the pentacetate of β -mannose.

The mother liquor from the crystalline pentacetate was distilled at 190°C . at a pressure of 0.01 mm. The distillate thus far did not crystallize. Its specific rotation was $+ 50^\circ\text{C}$., thus approaching very near that of the crystalline form.¹ It is possible that a very small amount of impurity prevents the crystallization of the remaining part of the pentacetate of α -mannose. However, it is equally possible that the syrupy mother liquor of the α -mannose contains a new isomeric pentacetate. If the latter possibility will be found to be true, then the explanation for the exceptional position of α -mannose with respect to the magnitude of the rotation of the carbon atom 1 will be self-evident. If, however, the former will be found to be the correct one, then the exceptional position of the α - and β -mannoses will require special explanation. One may be found in the assumption that the mannoses possess a structure different from the butyleneoxidic. However, for the present, few such sugars are known. Xyloses which supposedly have an amylenoxidic structure behave normally with respect to the value of the rotation of carbon atom 1. The third and fourth pentacetates of galactose also show a normal behavior in this respect. Thus, up to date, there is no evidence that the change in the ring formation within the sugar molecule leads to a change in the value of the optical rotation of the carbon atom 1. Besides,

¹ Since this paper was submitted for publication, the liquid distillate and a mother liquor from an additional experiment crystallized. The specific rotation of the crystalline material without further purification was $+ 50^\circ$.

exceptions from the van't Hoff superposition rule were observed in sugar derivatives which do not contain an oxidic structure as in the case of the amides or phenylhydrazides of monocarboxylic sugar acids. The exception to the van't Hoff superposition rule observed in several sugars by Hudson and Yanovski may be explained on the assumption that the optical rotation of a substance containing in its molecule more than one carbon atom is determined by two factors. One is the respective allocation of the radicles on the carbon atoms, the other is the orientation of carbon atoms with respect to one another within the molecule. The fact that the abnormalities are more pronounced in the free sugars and less in the pentacetates seems to lend support to this view.

EXPERIMENTAL PART.

Preparation of the Pentacetate of α -Mannose.—A solution of 160.0 cc. of pyridine and 125 cc. of specially purified acetic anhydride was chilled to 0°C.; in it 25.0 gm. of dry α -mannose were suspended and the mixture was allowed to stand in the refrigerator for 48 hours at 0°C. with occasional shaking. The reaction product was poured into a mixture of ice and water. An oil settled out. On repeated working with water containing ice the oil did not crystallize. Hence it was taken up in ether and worked free from pyridine first by very dilute hydrochloric acid, then by a weak bicarbonate solution, and finally with water. The ethereal solution was dried over sodium sulfate and the ether removed by distillation. The oil was then transferred into a dish and placed in a vacuum desiccator over sulfuric acid, and the desiccator placed in the cold room at 10°C. The oil was seeded by a few crystals of a pentacetate of mannose which we obtained through the courtesy of Dr. Hudson. After standing 4 days, the oil was thickly imbedded with crystals which could be removed by filtration. These crystals, which at this stage have a sticky character from adhering oil, were twice extracted with small portions of ether. After this operation the material acquired a dry character and on standing overnight in the desiccator over sulfuric acid, turned into a brittle mass. At this stage of purification the yield of the pentacetate was between 35 to 40 per cent of the employed sugar. The specific rotation of this material was $[\alpha]_D^{17} = \frac{+2.70^\circ \times 100}{2.5 \times 2} = +54^\circ$.

For further purification this material was extracted five times with ether. It then had the character of perfectly dry powder consisting of microscopic crystals. The specific rotation of this powder was $+ 57.6^\circ$. The melting point of the substance was 64°C . One part of the material was then recrystallized out of water and the other out of ether. Both had the same specific rotation.

$$[\alpha]_D^{17} = \frac{+ 2.88^\circ \times 100}{2.5 \times 2} = 57.6^\circ$$

The difference of the molecular rotations of the α and β forms is $(57.6 + 25.2) \times 390 = 32,292$. The corresponding value for the first and second pentacetates found by Hudson is 32,700.

The substance had the following composition.

0.1060 gm. substance: 0.1922 gm. CO_2 and 0.0550 gm. H_2O .

$\text{C}_6\text{H}_7\text{O}_6 (\text{COCH}_3)_5$. Calculated. C 49.21, H 5.68.

Found. " 49.04, " 5.80.

ISOMERIC METHYL DIACETONE MANNOSES.

By P. A. LEVENE AND G. M. MEYER.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, January 10, 1924.)

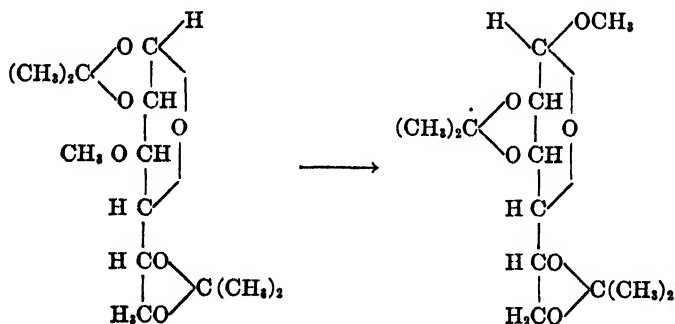
The knowledge of the structure of diacetone glucose was much advanced by the recent investigations of Levene and Meyer¹ and of Freudenberg and Doser.² It was natural to extend the investigations to the diacetone derivatives of other sugars. Work in this direction has been in progress in our laboratory. Meanwhile Freudenberg and Hixon³ have published a very interesting communication on the diacetone derivatives of galactose and mannose. From diacetone mannose they prepared a methyl derivative which showed a very peculiar behavior. By analogy with the methylation product of diacetone glucose the methyl group in methyl diacetone mannose was assumed to be in ether linking with carbon atom 3. As a rule, in methylated sugars, only the glucosidic methyl groups are readily hydrolyzed, all others being bound very firmly. Thus, 3-methyl-1,2-5,6-diacetone glucose is readily hydrolyzed into 3-methyl glucose. As a surprise it was found that in the methylation product of diacetone mannose the methyl group was cleaved off as readily as the acetone radicles. This observation is the first of its kind and if corroborated might have a far-reaching effect on the speculations regarding the structure of many polysaccharides. It is because of the importance of these problems that we are reporting now on a methyl diacetone mannose which was also prepared on methylation of mannose diacetone and which is not identical but isomeric with that obtained by Freudenberg and Hixon. These authors prepared their substance by methylation with sodium and methyl iodide. The product had a specific rotation of -41° . Our product was obtained by methylation by

¹ Levene, P. A., and Meyer, G. M., *J. Biol. Chem.*, 1922, liv, 805.

² Freudenberg, K., and Doser, A., *Ber. chem. Ges.*, 1923, lvi, 1243.

³ Freudenberg, K., and Hixon, R. M., *Ber. chem. Ges.*, 1923, lvi, 2119.

the method of Purdie and Irvine and had a specific rotation of $+23^\circ$. Also, in our product the methyl group was readily hydrolyzed and the product of hydrolysis was α -mannose. From the facts that the reactivity of the methyl group is the same in each isomer, and that the two differ only in their optical rotation, it is evident that the two isomers differ from one another in the manner of α - and β -glucosides. The question that needs to be answered is whether the acetone or the methyl group occupies the glucosidic position on carbon atom 1. The study of the model of mannose having a butyleneoxidic structure shows that it does not permit of the existence of α - β -acetone derivatives analogous to glucosides. In amylenoxidic and particularly in hexylenoxidic structures such isomers are possible. For mannose the possibility of the latter two structures is not excluded, but this theory offers no explanation to the behavior of the methyl groups. On the other hand, assuming the butyleneoxidic structure for mannose, the existence of α - β isomers of the methyl diacetone derivative may be explained only on the assumption of migration of the methyl groups from position 3 to 1 with a simultaneous transposition of one of the acetone unions from position 1 to 3.



A similar migration of groups was previously observed by the present writers in the case of 1-methyl-3,6-benzylidene glucose. On treatment with phosphorous oxychloride this derivative yielded a phosphoric ester of glucose which had the properties of 6-phosphoryl glucose. To test the theory of migration of the methyl group it was attempted to prepare a methyl diacetone mannose starting with methyl mannoside. Such a compound was prepared. It had practically the same boiling point as the

other methyl diacetone mannoses. The substance had the composition required by methyl diacetone mannose, the methyl value was only slightly below the one required by theory and the specific rotation was $+35^\circ$ as against $+23^\circ$ of one of the other methyl diacetone mannoses.

The discrepancy in the specific rotations of the two dextro-rotatory derivatives may be due either to structural differences of the two substances or to the fact that the substance obtained from diacetone mannose was not a pure α form, but contained a small proportion of the levo-rotatory isomer.

Thus for the present the theory of migration cannot be considered as conclusively established, but it is the more simple, and because of this, perhaps, the more probable of the two possible explanations. In this connection it is worthy of note that from diacetone glucose only one methyl derivative is formed regardless of the method of methylation.

It may be mentioned that the product obtained by the action on methyl mannoside contained besides the monomethyl diacetone still another substance which had a lower boiling point, decolorized bromine water, and had the composition of a methyl mannose condensed with 2 molecules of mesityloxyde. The true nature of the substance is for the present not known. It had the following composition. C = 63.84, H = 8.50, $\text{OCH}_3 = 9.22$, and was dextro-rotatory.

EXPERIMENTAL PART.

Methylation of Diacetone Mannose.

A. *Method of Purdie and Irvine.*⁴—20 gm. of mannose diacetone were dissolved in 60 gm. of methyl iodide and warmed to $60\text{--}65^\circ\text{C}$. in a glycerol bath. Silver oxide (30 gm.) was added in four portions during 2 hours. Then 40 gm. of methyl iodide were added and 40 gm. of silver oxide in five portions during 4 hours and heating continued for another hour.

The reaction product was extracted with anhydrous ether, which was then removed under diminished pressure and the resulting syrup, amounting to 20 gm., was subjected to fractional

⁴ Purdie, T., and Irvine, J. C., *J. Chem. Soc.*, 1903, lxxxiii, 1021.

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distillation. The main fraction boiled at 115° , $p = 1.2$ mm., and had the following optical rotation in acetylene tetrachloride.

$$[\alpha]_D^{20} = \frac{+0.60^{\circ} \times 100}{1 \times 2.58} = +23^{\circ}$$

It analyzed as follows:

0.1016 gm. substance: 0.2110 gm. CO_2 and 0.0723 H_2O .

0.1200 " " (Zeisel): 0.1034 gm. Ag I.

$\text{C}_{13}\text{H}_{22}\text{O}_6$. Calculated. C. 56.89, H 8.08, OCH_3 11.31.

Found. " 56.63, " 8.06, " 11.36.

Hydrolysis of Methyl Diacetone Mannose.

25 gm. of the syrup were hydrolyzed in 500 cc. of 50 per cent alcohol containing 2 gm. of HCl , in boiling water with reflux for 1 hour and 45 minutes. The concentration of the acid having been established volumetrically prior to hydrolysis, the acid was neutralized with the equivalent of 0.1 N NaOH . The solution was concentrated under diminished pressure at 35°C . and the residue extracted with absolute alcohol. This operation was repeated several times to remove inorganic material. The syrup which was obtained, eventually crystallized and analyzed as follows:

0.1002 gm. substance: 0.1446 gm. CO_2 , 0.0580 gm. H_2O , and 0.0022 gm. ash.

$\text{C}_6\text{H}_{12}\text{O}_6$. Calculated. C 40.00, H 6.66.

Found (ash-free). " 40.23, " 6.61.

The specific rotation of the substance in water was

Initial.	Equilibrium.
$[\alpha]_D^{20} = \frac{+1.24^{\circ} \times 100}{2 \times 2.5} = +24.8^{\circ}$	$[\alpha]_D^{20} = \frac{+0.84^{\circ} \times 100}{2 \times 2.5} = +16.8^{\circ}$

B. Method of Freudenberg and Hixon.—20 gm. of diacetone mannose were methylated by this process. The methylated product had the optical rotation in acetylene tetrachloride of

$$[\alpha]_D^{20} = \frac{-2.26^{\circ} \times 100}{1 \times 5.36} = -42.2^{\circ}$$

Methyl Diacetone Mannose from Methyl Mannoside.—90 gm. of methyl mannoside were shaken at 35°C. with 2 liters of dry acetone containing 1 per cent hydrochloric acid for 22 hours. Considerable undissolved material (80 gm.) remained which was filtered off. This was shaken again with 2 liters of acetone containing 1 per cent hydrochloric acid when again about 10 gm. had dissolved. This operation was repeated until 62 gm. had been dissolved. The acetone filtrates were each separately neutralized with sodium methylate and the filtrates combined and concentrated under diminished pressure to a thick syrup. This dark colored syrup was extracted with dry ether and filtered from inorganic material. The ether was removed under diminished pressure and the syrup which remained was extracted with 80–90° ligroin.

On removing this solvent under diminished pressure about 20 gm. of a light yellowish syrup were obtained which were subjected to fractional distillation. The first fraction boiled at 70–80°C., $p = 0.5$ mm., and had a liquid character. The succeeding fraction boiled at 105–112°C., $p = 0.5$ mm., and had the consistency of glycerol. This was again fractionated until a constant boiling material boiling at 105°, $p = 0.5$ mm., was obtained.

This analyzed as follows:

0.1060 gm. substance: 0.2204 gm. CO_2 and 0.0762 gm. H_2O .

0.1164 " " (Zeisel): 0.0952 gm. Ag I.

$\text{C}_{13}\text{H}_{22}\text{O}_6$. Calculated. C 56.89, H 8.08, OCH_3 11.31.

Found. " 56.63, " 8.04, " 10.79.

The optical rotation of the substance was

$$[\alpha]_D^{20} = \frac{+0.90^\circ \times 100}{1 \times 2.58} = +34.9^\circ$$

STUDIES OF AUTOLYSIS.

X. THE AUTOLYSIS OF MUSCLE.

BY K. K. CHEN AND H. C. BRADLEY.

(From the Laboratory of Physiological Chemistry, University of Wisconsin, Madison.)

(Received for publication, December 22, 1923.)

Hedin and Rowland (1) showed in 1901 that the press-juice of muscle contains a proteolytic enzyme, relatively weak, which acts in neutral, acid, and alkaline media. The digestion of the proteins in the press-juice goes less well in acid than in alkaline reacting media. So far as we are aware no other work has been published on the autolysis of muscle tissue as related to its H ion concentration, and it is evident that no conclusions can be drawn from the behavior of press-juice as to whole muscle tissue. Muscles are well known to undergo atrophy and under the same conditions which cause atrophy of epithelial organs; namely, a diminished blood supply. It was considered worth while, therefore, to study in some detail the autolytic behavior of muscle tissue and the effect which reaction has upon it. A preliminary report was made on this work in 1918 (2), in which it was stated that muscle tissues, striated, smooth, and cardiac, were found to autolyze at increased speed and to a greater extent in a slightly acid medium than in the control or alkaline digests. Our subsequent work has abundantly confirmed this result as far as mammalian tissue is concerned, but has brought out some striking anomalies in the behavior of muscle tissue from some of the lower animals.

In Tables I to IV we have selected a few typical cases from the large number collected¹ to illustrate the autolytic behavior of various types of muscles. The technique employed is the same as

¹ We desire to express our indebtedness to Drs. Sneeberger and Hattleberg, and Miss Gormley who collected much data confirming the findings reported here.

that used in previous studies. Autolysis is measured by amino acid production in trichloroacetic acid filtrates from the precipitated samples of tissue.

Warm Blooded, Striated Muscle.

TABLE I.
Dog Muscle.

No.	Condition.	0.2 N amino acids.						Net gain.	Autolysis.
		Days.							
		0	1	5	10	19	40		
		cc	cc	cc.	cc.	cc.	cc.		per cent
I	Control.....	0.10	0.25	0.55	0.60	0.85	0.95	0.85	7.7
II	“ +25 cc. 0.2 N HCl..	0.10	0.40	0.85	0.90	1.25	1.40	1.30	9.4
III	“ +50 “ 0.2 “ “	0.10	0.55	1.25	1.25	1.70	1.85	1.75	12.6
IV	“ +100 “ 0.2 “ “	0.10	0.10	0.35	0.30	0.40	0.45	0.35	2.5
V	“ +12.5 “ 0.2 “								
	NaOH.....	0.10	0.25	0.35	0.45	0.45	0.45	0.35	2.5
VI	Control +25 cc. 0.2 N								
	NaOH.....	0.10	0.25	0.35	0.30	0.40	0.45	0.35	2.5

Total N in 5 cc. brei.....11.05 cc. 0.2 N.

TABLE II.
Beef Muscle.

No.	Condition.	0.2 N amino acids.					Net gain.	Digestion.
		Days.						
		0	1	5	10	40		
		cc.	cc.	cc.	cc.	cc.	%	per cent
I	Control.....	0.15	0.25	0.30	0.40	0.45	0.30	2.16
II	" +5 cc. 0.2 N HCl.....	0.15	0.25	0.35	0.55	0.60	0.45	3.24
III	" +10 " 0.2 " ".....	0.15	0.25	0.50	0.75	0.80	0.65	4.68
IV	" +20 " 0.2 " ".....	0.15	0.30	0.55	0.90	1.10	0.95	6.85
V	" +30 " 0.2 " ".....	0.15	0.30	0.55	1.15	1.30	1.15	8.29
VI	" +50 " 0.2 " ".....	0.15	0.30	0.70	1.15	1.25	1.10	7.93
VII	" +100 " 0.2 " ".....	0.15	0.15	0.25	0.30	0.35	0.20	1.44
VIII	" +5 " 0.2 " NaOH.....	0.15	0.15	0.30	0.40	0.45	0.30	2.16
IX	" +20 " 0.2 " ".....	0.15	0.20	0.25	0.30	0.40	0.25	1.80
X	" +30 " 0.2 " ".....	0.15	0.15	0.25	0.35	0.35	0.20	1.44

Total N in 5 cc. brei.....11.10 cc. 0.2 N.

TABLE III.
Chicken Muscle.

No.	Condition.	0.2 N amino acids.				Net gain.	Digestion.
		Days.					
		0	3	7	40		
		cc.	cc.	cc.	cc.		per cent
I	Control (red muscle)	0.25	0.35	0.60	0.65	0.40	5.62
II	“ +25 cc. 0.2 N HCl.	0.25	0.30	0.85	0.95	0.70	8.22
III	“ +50 “ 0.2 “ “	0.25	0.50	1.00	1.20	0.95	10.39
IV	“ +75 “ 0.2 “ “	0.25	0.55	0.95	0.95	0.70	8.22
V	“ +100 “ 0.2 “ “	0.25	0.45	0.45	0.45	0.20	3.89
VI	“ +12.5 “ 0.2 “ NaOH.	0.25	0.20	0.20	0.30	0.05	2.60
VII	“ +25 “ 0.2 “ “	0.25	0.20	0.25	0.50	0.25	4.32

Total N in 6.25 cc. brei.

.12 cc. 0.2 N.

VIII	Control (white muscle)	0.25	0.35	0.45	0.45	0.20	3.48
IX	" +25 cc. 0.2 N HCl.	0.25	0.50	0.85	0.70	0.45	5.41
X	" +50 " 0.2 " "	0.25	0.50	1.00	0.85	0.60	6.57
XI	" +100 " 0.2 " "	0.25	0.35	0.50	0.40	0.15	3.09
XII	" +12.5 " 0.2 " NaOH	0.25	0.40	0.25	0.25	0.00	1.93
XIII	" +25 " 0.2 " "	0.25	0.40	0.45	0.25	0.00	1.93

Total N in 6.25 cc. brei.

.13.5 cc. 0.2 N.

TABLE IV.
Rabbit Muscle.

No.	Condition.	0.2 N amino acids.					Net gain.
		Days.					
		0	1	5	10	20	
		cc.	cc.	cc.	cc.	cc.	
I	Control.	0.20	0.20	0.30	0.30	0.65	0.45
II	“ +25 cc. 0.2 N NaOH.	0.20	0.20	0.25	0.30	0.55	0.35
III	“ +10 “ 0.2 “ “	0.20	0.25	0.25	0.25	0.55	0.35
IV	“ +5 “ 0.2 “ “	0.20	0.25	0.25	0.25	0.45	0.25
V	“ +5 “ 0.2 “ HCl	0.20	0.30	0.40	0.55	1.00	0.80
VI	“ +10 “ 0.2 “ “	0.20	0.80	0.47	0.70	1.20	1.00
VII	“ +25 “ 0.2 “ “	0.20	0.30	0.55	0.90	1.20	1.00
VIII	“ +50 “ 0.2 “ “	0.20	0.37	0.55	0.75	1.00	0.80
IX	“ +100 “ 0.2 “ “	0.20	0.25	0.25	0.25		
X	“ +150 “ 0.2 “ “	0.20	0.20	0.25	0.30	0.45	0.25

The data above show clearly that the usual mechanism determining autolysis in epithelial tissues is operative in striated muscle. The extent of digestion even under optimum conditions is, however, very much less. This may be explained in part as due to the relatively large mass of connective tissue present in striated muscle, which does not become substratum for the proteases present under the conditions of the experiment, nor under conditions met with in the body. We believe it is also indicative of structural proteins within the muscle fibers themselves which are not digested. This resistance of the muscle proteins to cleavage is a striking difference between it and epithelial tissues. To it we attribute the greater persistence of muscle cells undergoing atrophy as compared with many glandular atrophies. Another point of interest is the greater extent to which autolysis proceeds in the active pigmented muscle of fowl as compared with the less active unpigmented muscle tissue. There appears to be more potential substratum in the active tissue than in the inactive.

Warm Blooded, Cardiac Muscle.

TABLE V.
Pig Heart Muscle.

No.	Condition.	0.2 N amino acids.						Net gain.	Digestion.
		Days.							
		0	1	3	5	10	40		
		cc.	cc.	cc.	cc.	cc.	cc.		per cent
I	Control.....	0.20	0.40	0.55	0.60	0.95	1.00	0.80	6.53
II	“ +5 cc. 0.2 N HCl.	0.20	0.50	0.75	1.00	1.25	1.40	1.20	9.81
III	“ +10 “ 0.2 “ “	0.20	0.50	0.75	1.00	1.25	1.55	1.35	11.02
IV	“ +25 “ 0.2 “ “	0.20	0.55	1.00	1.30	1.50	1.65	1.45	11.84
V	“ +50 “ 0.2 “ “	0.20	0.65	1.00	1.20	1.30	1.60	1.40	11.43
VI	“ +75 “ 0.2 “ “	0.20	0.35	0.35	0.35	0.40	0.40	0.20	1.63
VII	“ +100 “ 0.2 “ “	0.20	0.20	0.25	0.25	0.30	0.35	0.15	1.22
VIII	“ +12.5 “ 0.2 “ “								
	NaOH.....	0.20	0.30	0.30	0.30	0.35	0.35	0.15	1.22
IX	Control +25 cc. 0.2 N								
	NaOH.....	0.20	0.25	0.30	0.30	0.30	0.30	0.10	0.9

Total N in 6.25 cc. brei.....12.25 cc. 0.2 n.

TABLE VI.
Beef Heart.

No.	Condition.	0.2 N amino acids.						Net gain.	Autolysis.
		Days.							
		0	1	3	5	10	40		
		cc.	cc.	cc.	cc.	cc.	cc.		per cent
I	Control.....	0.15	0.30	0.35	0.40	0.55	0.60	0.45	4.0
II	“ +5 cc. 0.2 N HCl...	0.15	0.25	0.55	0.65	0.70	0.85	0.70	6.2
III	“ +10 “ 0.2 “ “ ..	0.15	0.35	0.75	0.75	0.90	1.05	0.90	8.0
IV	“ +25 “ 0.2 “ “ ..	0.15	0.35	0.85	0.90	1.15	1.00	0.85	7.6
V	“ +50 “ 0.2 “ “ ..	0.15	0.40	0.75	0.85	1.05	1.00	0.85	7.6
VI	“ +100 “ 0.2 “ “ ..	0.15	0.15	0.15	0.20	0.20	0.25	0.10	0.9
VII	“ +5 “ 0.2 “ NaOH	0.15	0.25	0.30	0.30	0.35	0.40	0.25	2.2
VIII	“ +10 “ 0.2 “ “	0.15	0.20	0.25	0.25	0.30	0.35	0.20	1.8
IX	“ +20 “ 0.2 “ “	0.15	0.20	0.25	0.25	0.25	0.25	0.10	0.9
X	“ +30 “ 0.2 “ “	0.15	0.15	0.25	0.20	0.20	0.20	0.05	0.5
Total N in 6.25 cc. brei.....		11.05 cc. 0.2 N.							

Warm Blooded, Smooth Muscle.

Cardiac and smooth muscle are thus seen to behave like skeletal muscle. Acidity conditions autolysis while maintenance of neutrality or alkalinity inhibits digestion. Under optimum conditions autolysis is very small when compared with epithelial tissues.

TABLE VII.
Pig Stomach.

No.	Condition.	0.2 N amino acids.					Net gain.	Autolysis.
		Days.						
		0	1	5	10	40		
		cc.	cc.	cc.	cc.	cc.		per cent
I	Control.....	0.15	0.30	0.45	0.50	0.45	0.30	3.7
II	" +5 cc. 0.2 N HCl.....	0.15	0.30	0.55	0.60	0.60	0.45	4.9
III	" +25 " 0.2 " "	0.15	0.40	0.80	0.95	0.90	0.75	7.3
IV	" +50 " 0.2 " "	0.15	0.45	0.80	1.00	1.05	0.90	8.6
V	" +100 " 0.2 " "	0.15	0.20	0.30	0.50	0.25	0.10	2.0
VI	" +20 " 0.2 " NaOH...	0.15	0.20	0.20	0.35	0.30	0.15	2.4
VII	" +30 " 0.2 " " ...	0.15	0.15	0.20	0.20	0.20	0.05	1.6

TABLE VIII.
Beef Stomach.

No.	Condition.	0.2 N amino acids.				Net gain.	Autolysis.
		Days.					
		0	1	5	40		
		cc.	cc.	cc.	cc.		per cent
I	Control.....	0.25	0.35	0.55	0.55	0.30	4.5
II	“ +25 cc. 0.2 N HCl	0.25	0.30	0.50	0.55	0.30	4.5
III	“ +50 “ 0.2 “ “	0.25	0.30	0.60	0.70	0.45	8.8
IV	“ +100 “ 0.2 “ “	0.25	0.25	0.25	0.25	0.00	
V	“ +10 “ 0.2 “ NaOH.....	0.25	0.35	0.35	0.35	0.10	2.9
VI	“ +25 “ 0.2 “ “	0.25	0.25	0.35	0.35	0.10	2.9

pH Changes in Autolyzing Muscle.

In Table IX are given the changes in H ion concentration which muscle tissue undergoes during autolysis. In addition to the control, various reactions have been experimentally induced which cover the usual range in digestion experiments. It will be seen that the drift resembles similar changes in autolyzing liver brei (3). The reactions tend to converge toward a mean value of about pH 7—.

TABLE IX.
Rabbit Muscle.

No.	Condition.	pH									
		Days.									
		0	1	2	3	4	5	7	10	20	
I	50 cc. 0.2 N NaOH....	10.34	9.49	9.57	9.08	8.63	8.39	8.04	8.04		
II	25 " 0.2 " "	8.60	7.61	7.55	7.30	7.19	7.19	7.15	7.05	7.09	
III	10 " 0.2 " "	6.87	6.62	6.53	6.53	6.53	6.49	6.53	6.49	6.51	
IV	5 " 0.2 " "	6.40	6.28	6.29	6.28	6.23	6.29	5.25	6.26	6.33	
V	Control.....	5.73	5.85	5.87	5.91	5.92	5.95	6.03	5.91	6.03	
VI	5 cc. 0.2 N HCl.....	4.93	5.40	5.47	5.57	5.58	5.68	5.74	5.87	5.79	
VII	10 " 0.2 " "	4.35	4.87	5.19	5.20	5.30	5.41	5.44	5.27	5.52	
VIII	25 " 0.2 " "	4.02	4.15	4.31	4.42	4.50	4.68	4.77	4.79	4.83	
IX	50 " 0.2 " "	2.13	3.49	3.41	3.53	3.55	3.54	3.68	3.67	3.75	
X	100 " 0.2 " "	1.46	2.26	2.34	2.34	2.38	2.40	2.46	2.53	2.35	
XI	150 " 0.2 " "	1.02	1.68	1.75	1.79		1.72	1.72	1.54	1.53	

Smooth and cardiac muscle give essentially the same picture as striated muscle, though with less pronounced neutralization of the alkaline breis. In one case pig heart muscle developed the unusually high pH of 5.76 in 4 hours.

Cold Blooded Muscles.

While the warm blooded muscles have been characterized by a slow and small autolysis they have all behaved very much alike. In the group of cold blooded muscles examined, however, we have found some interesting variations. Frog striated muscle behaves very much like mammalian tissue. Muscles from the true fishes

TABLE X.
Frog Muscle.

No.	Condition.	0.2 N amino acids.					Net gain.	Autolysis.
		Days.						
		0	1	5	11	21		
		cc.	cc.	cc.	cc.	cc.		per cent
I	Control.....	0.15	0.35	0.55	0.60	0.70	0.55	7.2
II	" +10 cc. 0.2 N HCl.....	0.15	0.40	0.95	1.25	1.35	1.20	11.5
III	" +25 " 0.2 " ".....	0.15	0.55	1.10	1.35	1.65	1.50	14.4
IV	" +50 " 0.2 " ".....	0.15		0.90	1.15	1.35	1.20	11.5
V	" +100 " 0.2 " ".....	0.15		0.25	0.30	0.30	0.15	1.4
VI	" +25 " 0.2 " NaOH....	0.15	0.20	0.25	0.30	0.40	0.35	2.4
Total N in 6.25 cc. brei		10.44 cc. 0.2 N.						

(teleosts) show marked difference between species, and differ markedly also from muscles of the sharks (elasmobranchs). The muscles from lower cold blooded forms are still more strikingly different from mammalian.

Carp muscle evidently contains considerable potential substratum, as shown by the digestion in the presence of added acid. It does not, however, develop sufficient acidity post mortem to convert any of the muscle proteins into the digestible acid-protein form. The question arises, therefore, as to whether the carp ever undergoes atrophies of its musculature during normal life, or to what extent it can mobilize its proteins in fasting through an acidotic process, general or local. In the case of the salmon it

TABLE XI.
Perch Muscle; Average of Three Experiments.

No.	Condition.	Net gain in 10 days.
I	Control.....	0.15
II	" +6.25 cc. 0.2 N HCl.....	0.30
III	" +12.5 " 0.2 " ".....	0.55
IV	" +25 " 0.2 " ".....	0.80
V	" +50 " 0.2 " ".....	0.45
VI	" +125 " 0.2 " ".....	0.10

TABLE XII.
Carp Muscle.

No.	Condition.	0.2 N amino acids.						Net gain.	Autolysa.
		Days.							
		0	1	3	5	10	40		
		cc.	cc.	cc.	cc.	cc.	cc.		per cent
I	Control.....	0.30	0.35	0.35	0.30	0.30	0.30	0.00	2.5
II	" +10 cc. 0.2 N HCl.....	0.30	0.35	0.55	0.80	0.95	1.20	0.90	10.16
III	" +25 " 0.2 " ".....	0.30	0.45	0.90	0.90	1.35	1.50	1.20	12.70
IV	" +50 " 0.2 " ".....	0.30	0.50	0.70	1.10	1.35	1.55	1.25	13.12
V	" +100 " 0.2 " ".....	0.30	0.25	0.25	0.25	0.25	0.25	0.00	
VI	" +10 " 0.2 " ".....								
	NaOH.....	0.30	0.30	0.30	0.30	0.25	0.20	0.00	
Total N in 6.25 cc. brei.....		11.8 cc. 0.2 N.							

TABLE XIII.
Mackerel Muscle.

No.	Condition.	0.2 N amino acids.						Net gain in 22 days.
		Days.						
		0	1	2	5	11	22	
		cc.	cc.	cc.	cc.	cc.	cc.	
I	Control.....	0.20	0.75	0.85	1.60	1.78	1.95	1.75
II	" +10 cc. 0.2 N HCl.....	0.20	0.93	1.22	1.50	2.95	3.47	3.27
III	" +25 " 0.2 " ".....	0.20	0.95	1.15	1.40	2.50	2.95	2.75
IV	" +50 " 0.2 " ".....	0.20	1.02	1.30	1.90	2.57	2.90	2.70
V	" +10 " 0.2 " NaOH. .	0.20	0.35	0.33	0.40	0.62	0.83	0.63
VI	" +25 " 0.2 " "....	0.20	0.31	0.32	0.50	0.53	0.70	0.50

appears that during the long fast and migration, much of the muscle tissue itself is autolyzed and furnishes the necessary material for the maturing of the sperm and eggs. Whether the carp is able to call upon reserve protein from its muscle tissue would seem to depend largely on whether in fasting a considerable generalized acidosis develops. Postmortem acidity, if any develops, is insufficient to cause its muscle proteins to autolyze. We expect to obtain more data upon the behavior of carp muscle.

" TABLE XIV.
Menhaden Muscle.

No.	Condition.	0.2 N amino acids.						Net gain in 22 days.
		Days.						
		0	1	2	5	10	22	
		cc.	cc.	cc.	cc.	cc.	cc.	
I	Control.....	0.17	0.27	0.50	1.13	1.35	1.83	1.66
II	" +10 cc. 0.2 N HCl.....	0.17	0.46	0.88	1.85	1.90	2.65	2.48
III	" +25 " 0.2 " ".....	0.17	0.50	0.86	1.45	1.60	2.20	2.03
IV	" +10 " 0.2 " NaOH....	0.17	0.22	0.26	0.48	0.45	0.77	0.60
V	" +25 " 0.2 " ".....	0.17	0.25	0.30	0.45	0.50	0.75	0.58

TABLE XV.
Scup Muscle.

No.	Condition.	0.2 N amino acids.						Net gain in 21 days.
		Days.						
		0	1	2	5	10	21	
		cc.	cc.	cc.	cc.	cc.	cc.	
I	Control	0.10	0.10	0.10	0.17	0.24	0.35	0.25
II	" +10 cc. 0.2 N HCl	0.10	0.20	0.40	0.68	0.94	1.20	1.10
III	" +25 " 0.2 " "	0.10	0.15	0.30	0.50	0.90	1.10	1.00
IV	" +10 " 0.2 " NaOH	0.10	0.08	0.09	0.10	0.20	0.25	0.15
V	" +25 " 0.2 " "	0.10	0.09	0.09	0.09	0.10	0.27	0.17

In this series of salt water fish we find the most active fish, the mackerel, showing the largest autolysis in the control and in the acidified breis as well. While the data are perhaps too meager to draw any conclusions, it at least points in the same direction as the study of pigmented and non-pigmented fowl muscle; namely, that the more active the muscle the more potential substratum it contains and the more acid is produced post mortem also, converting some of the potential substratum into digestible form.

Shark muscle all runs strikingly below the autolytic figures from the bony fishes. The shark is rather sluggish and quickly tires when caught.

TABLE XVI.
Hammer Head Shark.

No.	Condition.	0.2 N amino acids.						Net gain in 20 days.
		Days.						
		0	1	2	5	10	20	
		cc.	cc.	cc.	cc.	cc.	cc.	
I	Control.....	0.35	0.35	0.35	0.50	0.61	0.70	0.35
II	“ +10 cc. 0.2 N HCl.....	0.35	0.43	0.45	0.57	0.74	0.85	0.50
III	“ +25 “ 0.2 “ “	0.35	0.40	0.47	0.58	0.75	0.90	0.55
IV	“ +10 “ 0.2 “ NaOH...	0.35	0.32	0.36	0.42	0.50	0.45	0.10
V	“ +25 “ 0.2 “ “	0.35	0.32	0.38	0.43	0.45	0.45	0.10

TABLE XVII.
Mackerel Shark Muscle.

No.	Condition.	0.2 N amino acids.				Net gain in 14 days.
		Days.				
		0	3	6	14	
		cc.	cc.	cc.	cc.	
I	Control..	0.10	0.25	0.30	0.40	0.30
II	" +25 cc. 0.2 N HCl. . . .	0.10	0.25	0.35	0.50	0.40
III	" +25 " 0.2 " "	0.10	0.20	0.20	0.20	0.10

TABLE XVIII.
Dog Fish Muscle.

No.	Condition.	0.2 N amino acids.					Net gain.
		Days.					
		0	1	3	9	20	
		cc.	cc.	cc.	cc.	cc.	
I	Control.....	0.25	0.30	0.40	0.60	0.65	0.40
II	“ +12.5 cc. 0.2 N HCl....	0.25	0.30	0.50	0.75	0.80	0.55
III	“ +25 “ 0.2 “ “	0.25	0.30	0.45	0.83	0.95	0.70
IV	“ +12.5 “ 0.2 “ NaOH..	0.25	0.30	0.30	0.35	0.40	0.15

In Tables XIX and XX are the autolytic data on muscles of two molluscs. *Sycotypus* is a carnivorous gastropod, slow of movement and evidently of low organization and metabolic

activity. The squid is the most active type of mollusc, a rapid swimmer, with well developed sense organs and nervous organization. It gives every evidence of a relatively high pitched metabolic rate.

In both forms muscle autolysis is very small, and but slightly increased by increased acidity. In these types we find no evidence of increased potential substratum in the more active muscle. It is

TABLE XIX.
Sycotypus Muscle.

No.	Condition.	0.2 N amino acids.				Net gain in 20 days.
		Days.				
		0	2	4	20	
		cc.	cc.	cc.	cc.	
I	Control.....	0.55	0.65	0.65	1.05	0.50
II	“ +40 cc. N acetic acid.....	0.55	0.65	0.65	1.20	0.65

TABLE XX.
Squid Muscle.

No.	Condition.	0.2 N amino acids.				Net gain.
		Days.				
		0	1	9	20	
		cc.	cc.	cc.	cc.	
I	Control.....	0.65	0.90	1.00	1.10	0.45
II	" +12.5 cc. 0.2 N HCl.....	0.65	0.95	1.35	1.35	0.70
III	" +12.5 " 0.2 " NaOH.....	0.65	0.90	0.95	1.05	0.40
IV	" +5 gm. CaCO ₃	0.65	0.85	1.05	1.10	0.45

doubtful whether atrophic changes in the muscles of these forms ever go on in the same functional way in which they occur in the salmon or in the mammal for example. Muscle proteins themselves appear to be too resistant to digestion to provide a substantial reserve of protein material for use by the animal, either as fuel during fasting, or for developing ova or sperm. We expect to subject this to further experimentation with the living animals, however.

DISCUSSION.

In general, we find that warm blooded muscle tissue autolyzes under much the same conditions as glandular tissue. It does not, however, digest to the same extent as glandular tissue. This difference is due, we believe, to the greater connective tissue content of muscle, and also to the presence within the muscle cells of structural proteins which are not affected by the cell proteases under any condition. Only a small fraction of the total muscle proteins are susceptible to atrophic hydrolysis. If the structural proteins digest it must be by an extremely slow process, or it may involve other enzymes than those found in muscle; for example, the enzymes of phagocytic cells. Muscle atrophy is always a slow process clinically, and corresponds thus with the reaction as found *in vitro*. Muscle tissue can evidently contribute through hydrolysis toward maintenance of the organism as a whole in prolonged fasting. But the slowness of the protein hydrolysis tends to conserve to the organism a structurally complete muscular machine.

In the muscles of the higher, warm blooded animals we find what seems to be a relation between activity, pigmentation, and autolysis. The more active muscles contain more protein susceptible of autolysis than the less active muscles. Thus the active leg muscles of the chicken, heavily pigmented with hemoglobin, yield more amino acids on autolysis than the inactive, unpigmented breast muscles. That the muscle protein fraction which is potential substratum is essential for contraction would seem to be indicated by the loss of contractile power in an atrophied muscle where the cells are still present and intact.

In some of the very active fishes muscle autolysis is more extensive than in the warm blooded animals, and much more so than in more sluggish fishes. Here again there appears to be a relation between the autolyzable protein fraction in muscle and the activity of the muscle. In fish like the mackerel a very considerable fraction of muscle protein could be mobilized for fuel or for growth of the sex structures. In the case of the salmon we know that extensive muscle atrophy takes place during the migration to the spawning beds (4). During this period no food is eaten, but the salmon swims great distances and at the same time matures

large quantities of ova or sperm. The proteins of the muscle tissue are very extensively mobilized during this period, and presumably by the autolytic mechanism.

In the more sluggish types of fish examined it does not appear that extensive atrophies can take place. In the single specimen of carp examined there proved to be potential substratum present, but in death the muscle cells developed so little acid that the proteins were not converted into substratum. If this is representative of the year round condition of the carp, it seems clear that only a generalized acidosis could make available for other uses the proteins of the musculature.

In the frog leg muscles we have about the same degree of autolysis as in the more sluggish types of fish. Nevertheless, the involution of the tadpole's tail is a striking example of complete removal of a mass of muscle tissue—a very unusual atrophy. This, we believe, cannot be referred to the autolytic mechanism alone, but must be accompanied by phagocytic action as well.

In the muscle tissue of molluscs we find a small autolysis developing, with very little increase by the addition of acid. Such muscles would appear incapable of more than a very slight atrophy indeed. Activity appears to make no difference in the degree of autolysis.

In the disuse atrophies of mammalian striated muscle we have loss of protein mass accompanied with very considerable loss of contractile power. With returning use, such muscles regularly hypertrophy again and the contractile strength is regained. This protein fraction, having a definite relation with contractile power, is, we believe, mobilized rather slowly as compared with gland proteins, but is, nevertheless, subject to similar fluctuations up and down. If acidity develops, this fraction is susceptible to autolysis and diminution. In severe and prolonged exercise accompanied with excessive fatigue, it is possible that a small amount of this contractile fraction is lost, so that the period for complete recovery may conceivably be conditioned by the time required to resynthesize it. We are subjecting this theory to more crucial tests by experimental atrophies of muscle in the living animal.

SUMMARY.

1. Warm blooded, striated, cardiac, and non-striated muscles are found to autolyze at increased speed and, to a greater extent,

in the presence of acids. The optimum concentration is usually between 0.04 and 0.02 N, or at a pH of about 4.5 to 5.0.

2. Under optimum conditions *in vitro* less than 15 per cent of muscle proteins digest to amino acids.

3. *In vivo*, atrophies of muscle tissue follow conditions which lead to acidosis of the muscle cells. Diminished blood supply from whatever cause produces such atrophic changes. Under ordinary conditions the atrophic changes are limited in extent and do not lead to death and removal of entire cells. Such limited atrophies may, however, result in almost complete loss of contractile power.

4. That fraction of muscle tissue which is found to be susceptible of autolysis appears to be particularly associated with the contractile function of the tissue. An atrophy of a few per cent of the total muscle protein mass, is accompanied by a disproportionately large loss of strength.

5. It is suggested that in prolonged excessive exercise and fatigue, sufficient acidity may develop in a muscle so that some of the contractile protein fraction is lost by autolysis. This may be a factor in determining the length of time required for complete recovery of strength from such excessive fatigue.

6. The intracellular proteases of muscle do not appear capable of completely digesting muscle tissue. Where muscle cells die it is believed phagocytic intervention is probably necessary for their complete removal.

7. In the types of fish muscle examined, the extent of autolysis or atrophy appears to be determined by the activity of the muscles. The more active, the greater the possible mobilization by autolysis of muscle proteins.

8. In the molluscs examined autolysis is slight, and practically unaffected by reaction. Activity does not alter the degree of autolysis.

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STUDIES ON EXPERIMENTAL RICKETS.

XXIV. THE EFFECT OF CERTAIN EXTRACTS OF PLANT TISSUES ON FLORID RICKETS.

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Observations by various investigators have demonstrated that certain fats contain a substance which exerts a pronounced beneficial effect upon the bones of growing animals (1). The best sources of this substance are cod liver oil and the liver oils of other fishes as the shark and burbot. Butter fat contains it in small amount. Of the vegetable fats and oils it has been clearly shown to be present only in coconut oil. The latter contains it only in very small amount, even as compared with butter fat. This substance, which it seems justifiable to class as a vitamin, is distinct from vitamin A (2). It has not as yet been found in any vegetable foods other than coconut oil.

The leaves of plants possess dietary properties superior in several respects to those of the storage organs (3). Also, it is a matter of common observation that grazing animals under natural conditions or under domestication rarely show skeletal defects provided they are given a satisfactory allowance of leguminous hays or grasses. On the other hand, rats which are confined to diets of cereal grains or to cereal grains and legume seeds always develop serious skeletal defects within a few weeks. This protective action of the leaf in the diet of the herbivorous animals may be due in great measure at least to the favorable relationships among the several mineral elements, especially of calcium and

phosphorus, which an abundance of the leaf in the diet would insure (4). On the other hand, the possibility remains that the leaf may furnish a sufficient amount of the vitamin which promotes the growth of normal bone to insure the development of a fairly good skeleton even when the other dietary conditions are such as would lead to faulty bone growth.

Therefore it has seemed desirable to examine some of the more important food plants in order to determine whether these contain the above mentioned vitamin. In this paper we give the result of feeding extracts of alfalfa, celery, carrots, spinach, brussels sprouts, cabbage, tomato, sweet potato, and clover.

We have tested lipin extracts of these vegetables for the anti-rachitic substance by observing whether they initiated healing in the bones of rats which were made rachitic by restricting them to our "line test" diet No. 3143. This diet has the following composition.

Ration 3143.

	<i>gm.</i>
Maize.....	33.0
Wheat.....	33.0
Wheat gluten.....	15.0
Gelatin.....	15.0
NaCl.....	1.0
CaCO ₃	3.0

The experimental diets differed from the formula which was employed for the production of rickets only in that the maize in the mixture carried the extract of the food under investigation.

The interval of time necessary for calcium to be deposited varies with the amount of the active principle or vitamin in the extract. 2 per cent of cod liver oil induces the deposition of a moderately heavy line of calcium salts in the proliferating cartilage of rachitic bones in 5 or 6 days (5). Preparations containing less of the vitamin cause the deposition of calcium salts at a slower rate, and in order to produce a calcification comparable to that formed under the influence of cod liver oil in 5 days it is necessary to extend considerably the time during which they are given. Tables I to XVII show the results of studies carried out on the above named vegetables. To each table is appended sufficient data to show how the material under investigation was prepared. The tables are self-explanatory.

TABLE I.

250 gm. of alfalfa meal were extracted with boiling ether under a reflux condenser for 2 hours. Eight portions of ether were applied in this manner. The ether extracts were filtered and combined, and mixed with the corn-meal to be used in making up the experimental ration, and the ether was distilled off, leaving the residue deposited upon corn-meal (330 gm.). This is the amount of corn-meal which was employed in making up 1 kilo of food of ration No. 3143. When the animals had acquired rickets on Diet 3143, the diet was made up anew according to the same formula, but with corn-meal which carried the extract of alfalfa instead of untreated meal. The results of including the ether-soluble matter of alfalfa in the rachitic diet, after rickets had been induced, are shown in Table I.

Lot No.	Rat No.	Days on diet.	Gain in weight.	Healing.	Remarks.
			gm.		
34	K-578	14		++	*
	K-59	14		++	
	K-76	21		++	
	K-79	21		++++	
	K-90	33		++++	
43	K-107	14		+++	
	K-118	19		+++	
	K-119	19		+++	
	K-120	19		?+	
	K-127	7		+	
53	K-136	16		++++	

* Since alfalfa contains a relatively large amount of phosphorus an ether extract was analyzed for phosphorus in order to see if the favorable results were due to an increased content of this element or to the antirachitic factor. The phosphorus was found to be negligible in amount.

TABLE II.

In Table II are shown the results of administering an alcoholic extract of alfalfa meal to rats suffering from experimental rickets. The alcoholic extracts were prepared in a manner similar to that described for the preparation of the ether extract. 1 kilo of ration contained the alcoholic extract of 250 gm. of alfalfa meal.

Lot No.	Rat No.	Days on diet.	Gain in weight.	Healing.	Remarks.
			gm.		
42	K-100	6		+	Very feeble.
	K-108	14		+++	
	K-121	19		+++	
	K-122	19		+++	
52	K-131	10		+++	
	K-141	24		+++	

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Animals which had been given extracts of alfalfa or of clover blossoms in their rations showed without exception healing rickets on postmortem examination. Many, when their bones were examined in gross at autopsy, showed an intense congestion of the

TABLE III.

In Table III are shown the results of feeding the acetone extract of alfalfa leaf to rats in the rachitic condition.

Lot No.	Rat No.	Days on diet.	Gain in weight.	Healing.	Remarks.
			<i>gm.</i>		
46	K-105	11		?	
	K-116	14		+	
54	K-137	17		++	
	K-139	20		++	
	K-140	20		++	

TABLE IV.

In Table IV are shown the results of feeding to rachitic rats the alcoholic extract of alfalfa leaves, made following the exhaustive extraction of the leaves with ether. The alcohol used was 95 per cent.

Lot No.	Rat No.	Days on diet.	Gain in weight.	Healing.	Remarks.
			<i>gm.</i>		
40	K-80	10		—	
	K-85	15		—	

TABLE V.

Alcoholic extract of 250 gm. of alfalfa which had been previously extracted with four portions of ethyl acetate. The extract was added to 1 kilo of Ration 3143.

Lot No.	Rat No.	Days on diet.	Gain in weight.	Healing.	Remarks.
			<i>gm.</i>		
57	K-144	13	-2	+	These animals were very weak when killed.
	K-145	13	+5	+	
	K-146	13	+9	+	

bones just shaftward of the epiphyseal cartilage. This congestion when present is characteristic of healing rickets in the rat. It may be so intense as to appear like hemorrhage. It can be seen in small bones without cutting them and in ribs, since it occurs in

TABLE VI.

125 gm. of alfalfa meal were boiled, first for about 20 minutes with 100 cc. of water. The water extract was then filtered off and 500 cc. of water were added and boiled for 20 minutes. This process was repeated two more times (four times altogether). The combined evaporated filtrates were added to 0.5 kilo of Ration 3143.

Lot No.	Rat No.	Days on diet.	Gain in weight.	Healing.	Remarks.
67	K-169	9	gm. +9	—	Fragment of calcium in metaphysis. Some calcium in metaphysis. Slight Ca in cartilage. Nutrition bad. Some spicules of calcification.
	K-177	12	+1	+	
	K-189	16	-2	+	
	K-196	21	-15	+	

TABLE VII.

125 gm. of dry clover blossoms were extracted under reflux condenser with four portions of ether (extracted about 3 hours). The extract was added to 0.5 kilo of Ration 3143.

Lot No.	Rat No.	Days on diet.	Gain in weight.	Healing.	Remarks.
64	K-166	9	gm. +5	+	Almost healed. Very marked healing.
	K-178	12	+1	++	
	K-186	16	0	+++	
	K-206	30	-5	+++	

TABLE VIII.

125 gm. of dry cabbage were extracted under reflux condenser for 3 hours with four portions of ether. The extract was added to 0.5 kilo of Ration 3143.

Lot No.	Rat No.	Days on diet.	Gain in weight.	Healing.	Remarks
68	K-170	9	gm. +7	—	Nearly healed. Doubtful. Some Ca in metaphysis. Doubtful, probably no healing.
	K-190	16	-3	+	
	K-191	16	+5	?	
	K-195	18	+3	?	

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TABLE IX.

125 gm. of dry cabbage were extracted under reflux condenser for 3 hours with five portions of ether. The extract was added to 0.5 kilo of Ration 3143.

Lot No.	Rat No.	Days on diet.	Gain in weight.	Healing.	Remarks.
			gm.		
77	K-216	7	+4	?	Probably no healing.
	K-221	14	+14	—	
	K-239	22	+7	—	

TABLE X.

Showing the effect of an ether extract of dried celery leaves on the healing of rickets in the rat. The procedure followed in making the extract was similar to that employed in making the ether extract of alfalfa. 125 gm. of dry celery leaves were extracted under reflux condenser with four portions of ether. This extract was added to 0.5 kilo of Ration 3143.

Lot No.	Rat No.	Days on diet.	Gain in weight.	Healing.	Remarks.
			gm.		
45	K-101	6		—	
	K-109	13		—	
	K-126	19		—	
	K-138	35		—	
59	K-155	9	+3	—	
	K-157	13	+19	—	
	K-183	30	+1	—	

TABLE XI.

In Table XI are given the results of ether extraction of 400 gm. of dried carrots. The procedure followed in making the extract was similar to that employed in making the extracts of alfalfa and celery. Both the celery and carrots were commercial products and we can say nothing of their treatment before they reached our laboratory.

Lot No.	Rat No.	Days on diet.	Gain in weight.	Healing.	Remarks.
			gm.		
38	K-65	13		—	
	K-66	13		—	
	K-69	18		—	
	K-83	23		—	
65	K-167	9	-3	—	
	K-187	16	+6	—	
	K-188	16	+4	—	
	K-204	30	+4	—	

the metaphysis just behind the costochondral junction. It gives the healing bones the appearance of bones in scorbutic

TABLE XII.

To show the effect on rickets of an ether extract of dry spinach. 125 gm. of dry spinach were added to 1 kilo of Ration 3143. The spinach was extracted under reflux condenser with four portions of ether.

Lot No.	Rat No.	Days on diet.	Gain in weight.	Healing.	Remarks.
			gm.		
56	K-142	7	+3	—	Found dead. Decomposition so far advanced that the rat was not completely autopsied.
	K-148	13	+5	—	
	K-151	19	+6	—	
	K-154	24		—	

TABLE XIII.

125 gm. of dry spinach were extracted under reflux condenser for 4 hours with five portions of ether. The extract was added to 0.5 kilo of Ration 3143.

Lot No.	Rat No.	Days on diet.	Gain in weight	Healing.	Remarks.
			gm		
73	K-211	8	+1	—	-
	K-215	15	+10	—	
	K-222	22	+5	—	

TABLE XIV.

125 gm. of dry brussels sprouts were extracted under reflux condenser for 3 hours with four portions of ether. The extract was added to 0.5 kilo of Ration 3143.

Lot No.	Rat No.	Days on diet.	Gain in weight.	Healing	Remarks.
			gm.		
69	K-171	9	0	—	.
	K-192	16	0	—	
	K-193	16	+3	—	
	K-205	30	+1	—	

animals. When the fresh bones were examined in silver nitrate solution they were found without exception to have reformed a

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provisional zone of calcification and to have a greater or lesser amount of lime salts deposited in the metaphysis.

TABLE XV.

125 gm. of dry tomato were extracted under reflux condenser for 4 hours with five portions of ether. The extract was added to 0.5 kilo of Ration 3143.

Lot No.	Rat No.	Days on diet.	Gain in weight.	Healing.	Remarks.
			<i>gm.</i>		
74	K-212	8	-6	—	
	K-217	15	+5	—	
	K-223	22	+6	—	

TABLE XVI.

Ether extract of 125 gm. of dry tomato was added to 1 kilo of Ration 3143. The tomato was extracted with four portions of ether under reflux condenser.

Lot No.	Rat No.	Days on diet.	Gain in weight.	Healing.	Itemarks.
			<i>gm.</i>		
55	K-143	7	+3	—	
	K-147	13	-5	—	
	K-150	19	+2	—	
	K-156	28	-4	—	

TABLE XVII.

125 gm. of dry sweet potato were extracted under reflux condenser for 4 hours with five portions of ether. The extract was added to 0.5 kilo of Ration 3143.

Lot No.	Rat No.	Days on diet.	Gain in weight.	Healing.	Remarks.
			<i>gm.</i>		
79	K-227	8	+4	?	
	K-241	16	+5	—	Probably no healing.
	K-242	16	-6	—	
	K-254	22	-9		Almost dead. Line of calcification due to fasting.

Sections were made from celloidin in blocks and stained with hematoxylin and eosin and with cresyl violet.

These sections showed that there was little or no alteration in the shafts of the bones. The trabeculae in the diaphysis were surrounded by board borders of osteoid tissue. The greater part of the cortex was also uncalcified. In the metaphysis, however, changes characteristic of healing could be seen to be taking place. Osteoid was being rapidly removed or calcified and normal bone was being formed about spicules of calcified intercellular substance which projected from the diaphyseal border of the disc of epiphyseal cartilage into the metaphysis. The blood vessels in the metaphysis were engorged with blood and new capillaries were making their appearance even in the osteoid tissue itself. The spaces between the blood vessels and the trabeculae were filled with hematopoietic tissue. The osteoid corpuscles could be seen to have undergone a complete metamorphosis. They had become round or oval cells with granular cytoplasm which stained deeply with basic dyes. Their nuclei were usually oval with a dense chromatin network. In the cytoplasm of each cell a large vacuole had formed which was about the size of the nucleus, and in fixed preparations contained a network of fine threads probably of some precipitated albuminous material. Many multinucleated giant cells (osteoclasts) were present in the areas in which reconstruction of bone was taking place.

Hart and Steenbock have studied the effects of dried leaves as contrasted with similar feeds in the fresh green state on calcium metabolism in herbivora, and found that persistent negative calcium balances which were maintained on dry rations were changed to positive calcium balances when preparations of fresh green food were given. The experiments here described lead us to conclude that the antirachitic substance is present in certain leafy foods even when the latter have been thoroughly dried. It is, of course, to be expected that curing the leaves should not be without influence on the special antirachitic nutritive quality as in the case of other nutritive principles.

It is of interest to note that among the vegetable tissues which we have studied thus far, the two products whose extracts showed an antirachitic effect were the legume plants—alfalfa and clover.

None of the bones taken from animals which had been treated with dried carrots showed any evidences of healing of the rickets. The same is also true of the bones of animals which had been

treated with celery, brussels sprouts, sweet potato, spinach, and tomato. It has been shown by McClendon and Shuck (6), Zucker and Barnett (7), and Goldblatt and Zilva (8) that extracts of spinach contain the vitamin A, but do not cure rickets.

The ether extract, of cabbage in most cases gave negative results. The one animal (No. K-190, Table VIII) which gave a decidedly positive test may be regarded with suspicion, since it was but one of seven, and there is a possibility that the animal may either have fasted just before being killed or that some error crept into the work.

It is interesting to note that tomato, which is known to be extraordinarily rich in the vitamin A, yielded extracts which were without antirachitic properties. This observation is in harmony with our experimental data reported elsewhere (2), which we interpreted to indicate that the antirachitic effect was due to a substance distinct from the vitamin A.

It would be very difficult to believe that the antirachitic effects of the extracts of clover and alfalfa could be due to the formation of insoluble calcium soaps in the intestinal tract of the experimental animals, and the consequent abstraction from the rachitic diet of absorbable calcium. The total amount of ether extract from these materials is small and consists only to a relatively small degree of fatty acids.

CONCLUSIONS.

1. Ether, alcohol, and acetone extract from alfalfa leaves a substance which causes healing of rickets in rats. The amount of extract used corresponded to 250 gm. of alfalfa meal per kilo of food. Healing began before the 7th day of administration and was practically complete in 33 days.

2. Ether completely extracts this substance from alfalfa, since alcoholic extracts of alfalfa which had previously been extracted with ether did not induce healing of rickets.

3. Ethyl acetate does not completely extract the antirachitic substance from alfalfa (Table V).

4. Extracts of alfalfa meal with boiling water are antirachitic.

5. The ether extracts of alfalfa are free from calcium and contain only insignificant traces of phosphorus.

6. Ether extracts the antirachitic vitamin from clover blossoms.

7. The results given by feeding ether extracts of cabbage were either negative or doubtful as regards the healing of experimental rickets.

8. Ether extracts of dry spinach, brussels sprouts, cabbage, celery, tomato, and sweet potato were given in amounts equivalent to feeding 250 gm. per kilo of ration to animals with rickets without any effect on the rachitic process in the bones. These vegetables, therefore, in a dried state are free from, or contain negligible amounts of, the antirachitic vitamin.

9. The healing of rickets which follows the administration of extracts of alfalfa or clover cannot be due to phosphorus or to the abstraction of calcium from the food by its precipitation in the intestine in the form of insoluble soaps of the therapeutic agent.

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STUDIES ON EXPERIMENTAL RICKETS.

XXV. A STUDY OF THE ANTIRACHITIC EFFECT OF CERTAIN OILS.

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In the following paper we present the results of a series of tests of the antirachitic effect of certain oils. The experimental technique was the same as that which we employed in the preceding communication.

It will be seen from an inspection of Tables I to XIV that the following oils showed no antirachitic effect, or only occasionally a suggestion of healing, which is probably to be attributed to fasting, since in these cases the loss of weight was excessive: Oil of sandalwood (Lot 85); oil of lemon (Lot 86); oil of orange peel (Lot 76); oil of palm (Lot 83); fraction of butter fat insoluble in alcohol (Lot 82); Japan wax (Lot 80); oil of spike (Lot 75); oil of fennel seed (Lot 66); spermaceti (Lot 62); and sperm oil (Lot 61).

The results of feeding sperm oil were rather surprising in that the rats all lost weight rather rapidly and died if restricted for any considerable period to diets containing this oil.

Rachitic animals had in some cases shown no signs of healing of the rachitic process notwithstanding the fact that they had lost very considerably in weight. This observation is of interest in view of the fact that prompt healing of rickets is induced by complete fasting of a rachitic animal. It would indicate that whatever may be the process which induces recalcification of rachitic bones during fasting, it is not dependent on loss of weight which occurs during the fasting period.

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TABLE I.

Lot 85. 10 gm. of oil of sandlewood were added to 500 gm. of Ration 3143* (2 per cent of the ration).

Rat No.	Days on diet.	Gain in weight.	Healing.	Remarks.
		gm.		
K-235	7	-11	+	Traces of healing.
K-250	10	-2	-	
K-257	15	-5	-	
K-281	22	+4	?	

* Ration 3143.

	gm.
Wheat.	33.0
Maize.....	33.0
Gelatin.....	15.0
Wheat gluten.....	15.0
NaCl.....	1.0
CaCO ₃	3.0

TABLE II.

Lot 86. 15 gm. of oil of lemon were added to 500 gm. of Ration 3143 (3 per cent of ration).

Rat No.	Days on diet.	Gain in weight.	Healing.	Remarks.
		gm.		
K-236	7	-11	+	
K-251	10	-9	-	
K-258	15	-9	-	
K-280	22	+2	-	

TABLE III.

Lot 76. 125 gm. of dry orange peel were extracted under reflux condenser for 3 hours with five portions of ether. Extract was added to 0.5 kilo of Ration 3143.

Rat No.	Days on diet.	Gain in weight.	Healing.	Remarks.
		gm.		
K-218	7	+1	-	Dead.
K-225	14	-5	-	
K-237	22	-14	-	
K-238	22	-7	-	

TABLE IV.

Lot 83. 15 gm. of oil of palm were added to 500 gm. of Ration 3143 (3 per cent of ration).

Rat No.	Days on diet.	Gain in weight.	Healing.	Remarks.
		gm.		
K-233	7	+3	—	Questionable.
K-248	10	+9	?	
K-255	15	+14	—	
K-282	22	+12	?	Questionable.

TABLE V.

Lot 82. 25 gm. of butter fat, free from alcohol-soluble matter, were added to 250 gm. of Ration 3143 (10 per cent of ration).

Rat No.	Days on diet.	Gain in weight.	Healing.	Remarks.
		gm.		
K-231	11	0	—	
K-232	11	—3	—	
K-245	14	—12	—	
K-246	14	—28	—	

TABLE VI.

Lot 80. 10 gm. of Japan wax were added to 500 gm. of Ration 3143 (2 per cent of ration).

Rat No.	Days on diet.	Gain in weight.	Healing.	Remarks.
		gm.		
K-228	8	+5	—	Found dead. Unable to tell anything about bones.
K-243	16	+11	—	
K-244	16	—1	—	
K-253	22	—22	?	

TABLE VII.

Lot 66. 125 gm. of ground fennel seed were extracted for about 3 hours under reflux condenser with five portions of ether. The extract was added to 0.5 kilo of Ration 3143.

Rat No.	Days on diet.	Gain in weight.	Healing.	Remarks.
		gm.		
K-168	9	+3	?	Fragments of Ca in tibia and cartilage.
K-182	16	+5	—	
K-198	23	+4	—	
K-208	30	+4	—	

TABLE VIII.

Lot 62. 20 gm. of spermaceti were incorporated into 1 kilo of Ration 3143 (2 per cent of ration).

Rat No.	Days on diet.	Gain in weight.	Healing.	Remarks.
		<i>gm.</i>		
K-160	8	+3	—	Almost dead. Probably fasting, although food in stomach.
K-164	15	—1	—	
K-174	21	—11	+	

TABLE IX.

Lot 61. 30 gm. of sperm oil were added to 1 kilo of Ration 3143 (3 per cent of ration).

Rat No.	Days on diet.	Gain in weight.	Healing.	Remarks.
		<i>gm.</i>		
K-159	8	—17	++	Fasting?
K-163	15	—6	—	Died. Some calcification. Almost dead. Results unsatisfactory. It is possible that fasting may have occurred at some time during experiment.
K-172	21	—13	?	
K-179	23	—11	++	

TABLE X

Lot 63. 20 gm. of oil of spike were incorporated in 1 kilo of Ration 3143 (2 per cent of ration).

Rat No.	Days on diet.	Gain in weight.	Healing.	Remarks.
		<i>gm.</i>		
K-161	8	+1	+	Almost dead. Practically healed. ?
K-165	15	—23	+	
K-176	21	—19	++	
K-185	25	—17	—	

TABLE XI.

Lot 75. 5 gm. of oil of spike were added to 500 gm. of Ration 3143 (1 per cent of ration).

Rat No.	Days on diet.	Gain in weight.	Healing.	Remarks.
		<i>gm.</i>		
K-213	8	+8	—	
K-219	15	—2	—	
K-224	22	+7	—	

Animals which received oil of cloves as an addition to the rachitic ration uniformly showed an intense degree of healing. The addition of 2 per cent of this oil to the rachitic diet was sufficient to initiate prompt recalcification of the rachitic bones. In no

TABLE XII.

Lot 71 A. 15 gm. of alcoholic extract of butter fat were added to one-half kilo of Ration 3143 (3 per cent of ration). The total amount of the alcoholic extract from 5 lbs. of butter was about 40 gm.

Rat No.	Days on diet.	Gain in weight.	Healing.	Remarks.
		<i>gm.</i>		
K-181	4	+1	—	
K-207	20	+14	++	

TABLE XIII.

Lot 71 B. 25 gm. of alcoholic extract of butter fat were added to 250 gm. of Ration 3143 (10 per cent of ration). The total amount of the alcoholic extract from 5 lbs. of butter was about 40 gm.

Rat No.	Days on diet.	Gain in weight.	Healing.	Remarks.
		<i>gm.</i>		
K-229	11	+2	++	
K-230	11	+7	++	

TABLE XIV.

Lot 84. 10 gm. of oil of cloves were added to 500 gm. of Ration 3143 (2 per cent of ration).

Rat No.	Days on diet.	Gain in weight.	Healing.	Remarks.
		<i>gm.</i>		
K-234	7	-10	+	
K-249	10	+6	++	Almost healed.
K-256	15	-5	++	
K-273	19	-20	+++	
K-290	8	-2	++	Dead when found. Some had been eaten.
K-289	8		++	" " " "

case was the result due to fasting since the animals ate greedily up to the time of death. Nor can the healing be explained by tissue destruction in the body of the animals, although many of these animals lost a considerable amount of weight. Rat K-249, which

gained 5 gm. during the 10 days over which the oil was administered, showed quite as advanced healing as that which was demonstrable in the bones of animals which lost weight during the experimental period.

When the yellow oil of butter (alcoholic extract of butter fat) was added to the rachitic ration to the amount of 3 per cent a relatively long period elapsed before marked signs of healing occurred. When the extract was given to the amount of 10 per cent the animals showed advanced healing after the administration of the oil for 11 days. All the animals which received this oil gained in weight during the experimental period.

Of the four animals to which we fed oil of spike to the amount of 2 per cent of the ration, No. K-185 showed no trace of healing in spite of a loss of weight of 17 gm. during the 25 days it received the oil. The other three animals showed evidence of healing under the influence of the oil. Two of these animals lost a great deal of weight, 23 and 19 gm., respectively, and one was in an extremely poor condition when it was killed. The fourth animal, No. K-161, showed healing of the bones, and gained 1 gm. in weight during the 8 days of treatment.

When 1 per cent of oil of spike was administered the results were uniformly negative as regards healing of the rachitic bones, even after 22 days of treatment. It is difficult to believe that the effect of 2 per cent of this oil in the healing of rickets was the result of the activity of the antirachitic vitamin. If such were the case one would expect that since 2 per cent of the oil caused healing in 8 days, 1 per cent should have initiated it in 22 days, which was not the case. It is more likely that doubling the dosage of this oil was sufficient to induce healing through the agency of something which caused effects analagous to those which are produced by complete fasting of the animal. This is supported by the weight charts which show that, while the animals receiving 2 per cent of the oil with one exception lost large amounts of weight, the loss among animals receiving 1 per cent was negligible, and in fact in two instances out of three they gained in weight.

To sum up the results of this paper it may be said that alcoholic extracts of butter fat apparently contain appreciable amounts of the antirachitic substance, and that the same substance is present in oil of cloves.

STUDIES ON ENZYME ACTION.

XXV. COMPARATIVE LIPASE AND PROTEASE ACTIONS OF THE FLEXNER-JOBLING RAT CARCINOMA AND OF DIFFERENT RAT TISSUES.

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INTRODUCTION.

According to the generally accepted view of enzyme actions, a definite connection exists between such actions and the chemical changes occurring in life processes (1). Possibly, enzymes control these changes; at the very least, there is an interdependence. The investigation in progress involves the comparative study of the enzyme actions of abnormal growths such as tumors with the enzyme actions of normal tissues and organs. Some of the results, mainly preliminary in character, have been presented in the earlier papers of this series (2-5).

A systematic study of the enzyme actions of animal tissues and tumors must be based upon a suitable choice of materials and actions in order that the results may be interpreted in a definite manner. The choice of the enzyme actions to be studied was controlled by the following conditions.

The chemical change to be as definite as possible.

The chemical manipulation to be as simple as possible, since, in view of the nature of the materials, it was necessary to carry out a large number of determinations within a relatively short space of time.

The possibility of varying the substrate in a more or less continuous, progressive, or systematic manner, since it is to be expected that

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certain of the tissues and tumors would show small differences in actions.

The conditions imposed limited the study, in the main, to the ester-hydrolyzing enzymes of the materials, and a comparison of the actions of the tissues and tumors on a number of different esters. A peptone and a casein preparation were also studied in a number of cases in order to determine whether the protease actions paralleled the lipase actions. The results of these protease actions cannot be interpreted as simply as the lipase actions, because of the chemical complexities of the substrates.

With reference to the enzyme material, it may be pointed out that tumors of human origin are obtainable with difficulty in forms suitable for continuous laboratory study. It was necessary, therefore, to use material from a different source for the initial study. The Flexner-Jobling rat carcinoma, available in quantity, and of more or less constant properties, was used for the determination of the fundamental characteristics of the enzyme actions of a definite tumor type. The enzyme actions of the various tissues of the rat were studied similarly. The results so obtained are presented in this paper. In subsequent papers, the results obtained in the study of the corresponding tissues of other animals and of different tumor types will be presented, and later, similar studies on various groups of tumors of human origin and on some human tissues will be given.

Historical.

The different actions of a definite enzyme preparation upon a number of substrates and the actions of a number of different enzyme preparations upon the same substrate have been studied frequently in the past, although in some of the earlier work the experimental conditions were not carefully controlled. These two methods of study are involved in the work to be described here. The different actions observed are generally included under the terms of specificities of enzyme actions or selective actions of enzymes.

It is inadvisable to attempt to give here a complete list of such actions. Only some of these, more directly related to the present work, will be mentioned.

Hanriot (6) in 1897 found that the action of blood serum on monobutyrin differed from that of pancreas. More extended investigations of Kastle and Loevenhart (7), of Loevenhart and Peirce (8), and of Loevenhart (9) involved the actions of pancreas and liver extracts of different animals on a number of esters under varying conditions. Definite differences in the actions were recorded and regularities with different esters noted. Chanox and Doyon (10) found that liver lipase hydrolyzed amyl salicylate, while pancreas and blood serum did not. The studies of Abderhalden and his associates (11), begun in 1906, included the actions of various extracts on synthetic polypeptides as well as of definite tissues on peptones prepared from proteins of the same tissues, which were found to be hydrolyzed while no action was observed on peptones prepared from proteins of different tissues. Armstrong and Ormerod (12) studied the different actions of liver, stomach, pancreas, and castor bean lipases on natural fats and on esters. An esterase and a lipase were shown to occur in castor beans (13) and also in human intestinal contents, the former in the succus entericus, the latter in the pancreatic juice and bile (14), as a result of the comparative actions on glyceryl triacetate and ethyl butyrate. This list might be extended, but enough work has been quoted to show the nature of the studies carried out, although, because of different conditions of experimenting, the results are not always readily comparable.

Experimental Methods.

In deciding which esters were to be used as substrates for the lipase or ester-hydrolyzing actions, it was evident that it would be difficult to obtain reproducible conditions and comparable results with insoluble esters such as the natural fats. The formation of an emulsion, the presence of substances to stabilize such emulsions, and the continuous agitation of the mixture seem necessary conditions for working with these. Results will be given in this paper for the following esters: Methyl acetate, ethyl acetate, isobutyl acetate, phenyl acetate, benzyl acetate, glyceryl triacetate, methyl butyrate, ethyl butyrate, methyl benzoate, and ethyl benzoate. Three pairs of isomeric esters are included in the list. 3.4 milli-equivalents (an arbitrary, but convenient, quantity) of ester were used in each experiment. Aside from gross impurities, which were readily removable, the impurities of the esters were tested by incubation with water at 38° for 22 hours. Negligible amounts of acid are produced, between 0.004 and 0.012 milli-equivalents as judged by titration with 0.1 N alkali.

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For the protease actions, a peptone preparation (Fairchild Brothers and Foster) and a casein preparation (purified according to Hammarsten), 0.1 gm. of each in each test, were used.

The enzyme material was obtained from albino rats, both male and female, fed on white bread soaked in whole milk, fresh cabbage or carrots, and tap water *ad libitum*. The tumor material was obtained as a result of inoculation with the Flexner-Jobling rat carcinoma in the neighborhood of the right axilla in the usual way (15) and permitting growth for from 3 to 6 weeks. The neoplasm was removed from the animal after killing it with ether according to the method described in detail by Sugiura and Benedict (15). The non-neoplastic tissue was removed, the tumor material cut into small pieces or macerated with sand, and water added for extraction. The rat tissues were obtained either from normal rats or tumor-bearing rats as soon after killing with ether as possible. They were ground as fine as possible and extracted with water. The quantity of water added in each case depended upon the approximate concentration of material to be tested. Tests showed that water extraction gave very nearly the same results as extraction with physiological salt solution, so that only the former was used. Toluene was added at once, the mixtures were kept overnight in an ice box and filtered through paper the next day. Portions of 5 cc. each of the filtrates were diluted with water to 15 cc. as a rule, and tested. In a number of cases, portions of the solid residues after filtration were weighed out, 15 cc. of water added, and tested similarly.

The tissues from both normal rats and tumor-bearing rats were tested because of the possibility of the presence of the tumor influencing the actions of the tissues. Such an influence was observed only with the liver and may well have been due to metastases, as will be shown later.

The material for each series of experiments was obtained from six to twelve rats.

The amounts of lipase actions were determined by titration with 0.1 N sodium hydroxide solution, with phenolphthalein as indicator. For the protease actions, the formol method with phenolphthalein and 0.1 N sodium hydroxide solution was used. Previous work (3) had shown that the formol and Van Slyke

methods gave similar results. Corrections were introduced in every case for the enzyme material and substrate (ester or protein) blanks. Toluene was present throughout the experiments. Each test was made in duplicate.

As stated, the volumes of the solutions tested were 15 cc. The experiments were run at 38° for 22 hours. This time was chosen as most suitable as a result of the experiments on the kinetics of the actions (5). For most of the materials the rapid hydrolyses had ceased at this time, but the actions had in no way approached completion.

The solutions were all brought to pH 7.0 initially. Because of the acid production in the ester hydrolyses, they dropped to pH 5.0 to 5.5 comparatively rapidly (much more rapidly in some cases than in others) and then remained fairly constant. Experiments carried out with the mixtures at pH 5.0 initially gave the same relative actions on the different esters as when started at pH 7.0, but smaller absolute actions. In order to obtain more satisfactory comparisons, therefore, all the actions which will be shown were obtained with the mixtures initially at pH 7.0.

It is obvious, of course, that a truer comparison of the actions would be obtained if the times for equivalent actions were taken in place of the amounts of action in equal times. Practical difficulties, due in part to the nature of the experiments, and in part to the uncertain application of the kinetics of the different actions, made it advisable to use the theoretically less accurate method of determining and presenting the results.

Presentation of Results.

Because of the large amount of data at hand, it would be impossible, even if desirable, to give the experimental results in detail. For practical reasons they must be grouped and summarized.

Two methods of presenting the results are available. In the first instance they may be given as the absolute actions of each preparation or material on the various esters. Secondly, the results for the actions of any one enzyme preparation on a number of esters may be given as relative actions; that is, denoting the

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greatest action by 100, and calculating the actions on the remaining esters in that series in terms of this. It will be seen that the second method of presentation is perhaps the more significant, although for a satisfactory understanding of the relations, both methods must be employed.

In Table I are shown the absolute amounts of esters hydrolyzed by the tumor and various tissues of the rat in some of the experiments. These were selected in order to indicate the magnitudes of the actions for various concentrations of the enzyme materials. In some cases the amounts of actions for the same concentration of a given tissue in different series differed considerably. Such variations in absolute actions are unavoidable when dealing with living matter.

The data in the table are self-explanatory. In Column 1, the letters *RT* with the number of the experiment signify that the rats from which the indicated tissue was obtained were tumor-bearing.

The results show also the relative accuracy obtainable in the different series and within any one series. For example, a comparison of two absolute actions of 0.3 each or less signifies little as to their relative actions in a series although a comparison of such actions with a much larger absolute action would be of importance.

A separate discussion of the results of Table I is unnecessary.

Following Table I are shown a number of charts in which the relative actions on the various esters, as percentages of the greatest action in that series, of the different tissues are presented. The absolute actions found are not involved directly in these charts although, of course, the relative actions are calculated from them.

The results for the tumor and different tissues shown in Table I and Figs. 1 to 16 may be discussed briefly. The order in which the esters are plotted as abscissa is entirely arbitrary. The same order is used in every plot. The ordinates show amounts of hydrolysis in terms of percentage action upon equivalent amounts of the indicated esters which are plotted at equidistant intervals on the abscissa axis.

TABLE I.

Hydrolyzing Actions in Tenths of Milli-Equivalents of Acid Produced by Rat Tissues and Carcinoma on the Indicated Esters.

Experiment No.	Tumor or tissue extracted per cc. solution tested.	PhOAc	Cl(OAc) ₂	MeOBu	PhCH ₂ OAc	EtOAc	MeOAc	EtOBu	MeOBz	EtOBz	IsobuOAc
Flexner-Jobling rat carcinoma extracts.											
	mg.										
97	8.9	1.86	1.18	0.37	0.28	0.23	0.24	0.31	0.00	0.00	0.30
100	17.8	2.84	1.95	0.64	0.48	0.49	0.46	0.50	0.10	0.08	0.46
91	53.4	5.31	4.08	1.38	1.12	1.11	1.15	1.08	0.19	0.15	0.91
88	78.5	6.01	4.45	1.75	1.43	1.48	1.36	1.44	0.25	0.24	1.16
84	89.2	7.94	5.55	2.05	1.65	1.68	1.69	1.11	0.22	0.31	1.38
Carcinoma residues after extraction.											
79	33.3	3.61	2.33	0.77	0.35	0.30	0.33	0.18	0.10	0.04	0.00
84	66.7	5.01	4.31	1.41	0.74	0.78	0.80	0.30	0.06	0.04	0.47
Leg muscle extracts.											
95 B	8.9	0.38	0.18	0.17	0.13	0.08	0.05	0.22	0.00	0.08	0.00
95 A	65.7	2.16	1.15	1.74	1.20	0.98	0.72	1.83	0.33	0.67	0.76
84 RT	59.7	2.01	1.18	2.01	1.06	0.99	0.82	1.04	0.45	0.66	0.91
64 RT	96.4	2.81	1.81	2.77	1.54	1.43	1.11	1.81	0.95	1.06	(0.33)
Leg muscle residue after extraction.											
88 RT	66.7	2.19	0.81	2.04	0.35	0.64	0.40	1.39	0.27	0.32	0.29
Heart muscle extracts.											
65	11.9	1.00	0.57	1.10	0.36	0.44	0.28	0.27	0.14	0.31	0.00
63	15.2	1.38	0.76	2.01	0.55	0.58	0.40	0.68	0.38		
91 RT	6.1	0.97	0.36	0.92	0.13	0.28	0.12	0.62	0.12	0.22	0.07
64 RT	11.4	1.17	0.61	1.26	0.35	0.42	0.26	0.32	0.25	0.34	0.00
Heart muscle residue after extraction.											
84 RT	66.7	1.71	1.04	1.55	0.22						
Kidney extracts.											
95	8.9	8.64	5.56	3.64	3.06	4.88	2.53	6.09	0.57	0.92	
63	38.9	12.57	9.71	6.96	4.69	7.37	4.63	7.07	1.40		
91 RT	17.7	11.43	7.60	4.84	3.93	5.84	3.80	5.76	0.90	1.24	4.92
64 RT	29.7	13.71	9.14	5.26	4.30	6.26	3.15	4.91	1.11	1.62	4.31

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TABLE I—Continued.

Experiment No.	Tumor or tissue extracted per cc. solution tested.	PhOAc	Cl(OAc) ₂	MeOBu	PhCH ₂ OAc	EtOAc	MeOAc	EtOBu	MeOBz	EtOBz	IsobuOAc
Kidney residues after extraction.											
	mg.										
81 RT	16.7	7.53	5.26	4.39	3.00	5.26	2.90	2.50	0.51	1.05	3.05
84 RT	26.7	9.15	7.14	4.94	3.98	5.91	4.11	3.16	0.91	0.86	4.29
Lung extracts.											
95	8.9	3.56	1.82	5.29	1.10	2.05	1.43	(5.66)	0.72	1.16	0.79
100 RT	8.9	3.40	1.82	4.79	0.69	1.42	1.07	3.92	0.77	0.98	0.97
91 RT	15.1	4.39	2.40	6.23	1.19	2.41	1.77	4.41	1.46	1.32	1.47
88 RT	20.4	4.50	2.52	6.29	1.28	2.64	2.01	4.05	1.35	1.27	1.76
Lung residues after extraction.											
88 RT	16.7	3.56	1.31	4.36	0.42	1.23	0.88	2.77	0.70	0.72	0.75
84 RT	33.3	4.47	2.28	5.80	0.91	2.26	1.93	2.41	0.96	0.88	1.26
Spleen extracts.											
95	8.9	5.58	3.79	4.90	0.61	0.56	0.76	0.81	0.00	0.00	0.31
63	25.8	9.59	7.22	8.30	1.32	1.32	1.51	1.40	0.42		
100 RT	8.9	5.30	3.55	3.67	0.53	0.55	0.76	0.46	0.05	0.03	0.44
75 RT	16.6	7.64	5.28	5.06	0.79	0.97	1.04	0.66	0.09	0.04	0.13
Spleen residues after extraction.											
79 RT	16.7	3.73	2.45	3.46	0.19	0.20	0.19	0.34	0.05	0.00	0.00
84 RT	33.3	5.81	4.20	7.47	0.47	0.56	0.75	1.07			
Testes extracts.											
95	8.9	7.06	4.04	(2.92)	0.79	1.38	1.16	3.74	0.55	0.72	
65	35.3	11.30	8.10	7.17	1.93	4.55	3.21	4.82	1.49	1.67	1.64
91 RT	19.6	8.76	5.95	5.49	1.11	2.30	1.96	4.18	1.23	1.16	1.27
88 RT	26.5	8.72	6.16	7.20	1.65	3.20	2.75	5.20	1.41	1.36	1.78
Testes residues after extraction.											
83 RT	16.7	4.32	1.88	4.96	0.47	1.42	0.76	2.20	0.63	1.21	(0.31)
84 RT	33.3	6.54	3.49	5.54	1.26	2.62	2.40	2.87	1.14	1.12	1.38

TABLE I—*Concluded.*

Experiment No.	Tumor or tissue extracted per cc. solution tested.	PhOAc	Cl(OAc) ₂	MeOBu	PhCHOAc	EtOAc	MeOAc	EtOBu	MeOBz	EtOBz	IsobuOAc
Brain extracts.											
	<i>mg.</i>										
95	8.9	1.33	0.79	0.04	0.20	0.15	0.11	0.00	0.00	0.00	0.00
67	21.6	1.85	1.17	0.24	0.32	0.30	0.27	0.00	0.00	0.00	0.00
91 RT	23.2	2.62	1.86	0.39	0.53	0.44	0.45	0.19	0.00	0.02	0.37
88 RT	32.1	3.18	2.07	0.43	0.61	0.48	0.51	0.25	0.00	0.04	0.40
Brain residue after extraction.											
85 RT	66.7	2.80	2.45	0.56	0.61	0.57	0.55	0.16	0.02		
Liver extracts.											
95 B	8.9	7.66	4.40	4.87	2.10	2.58	1.61	4.46	0.61	0.82	
95 A	52.3	11.00	10.50	10.62	4.16	7.35	5.27	8.22	1.76	1.79	5.52
63	93.3	12.54	10.78	11.93	5.48	9.02	6.58	8.91	2.84	2.39	5.52
97 RT	8.9	9.39	6.21	7.15	4.14	4.30	2.66	7.04	0.85	1.19	4.41
91 RT	58.0	14.27	11.88	11.38	7.46	8.81	6.95	9.13	2.59	2.29	7.30
76 RT	87.7	14.09	11.64	10.45	4.50	6.98	5.40	6.81	2.29	1.91	4.69
Liver residues after extraction.											
84 RT	33.3	10.48	8.96	9.67	6.67	8.43	7.41	6.13	1.61	1.76	5.98
88 RT	50.0	9.80	9.49	12.14	5.26	8.84	5.92	9.09	1.79	1.86	7.01

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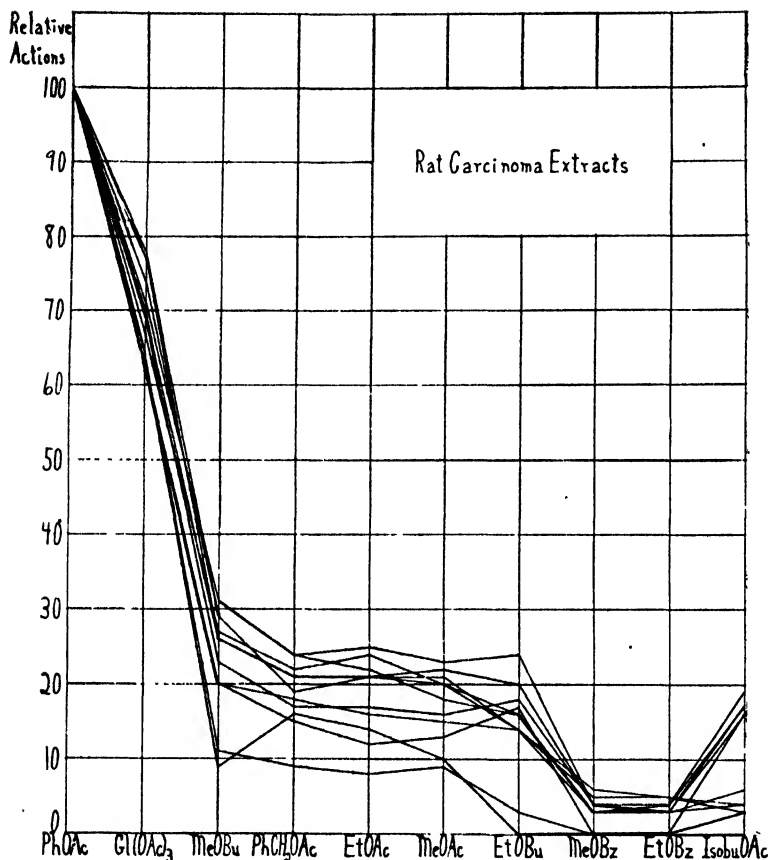


FIG. 1. Carcinoma extracts. Each curve represents the results of one series with extracts of tumors grown for 3 to 4 weeks in from six to twelve rats. The curves coincide quite closely when it is considered that in addition to the errors incidental to the various manipulations, very different amounts of tumor were extracted in the different series. There is evidently a definite relation between the actions on the various esters, not always clearly indicated when two esters only are considered, but shown definitely by the general nature of the complete curves. With isobutyl acetate and ethyl butyrate two sets of results are apparent. This is due to the fact that in the later experiments purer esters were used showing greater actions. As for a more detailed discussion it may be pointed out that with PhOAc taken as 100, Gl(OAc)₃ ranged from 63 to 78, and the other esters less than 30. No definite differences were observable between the corresponding methyl and ethyl esters, while PhCH₂OAc was not much different from MeOAc and EtOAc.

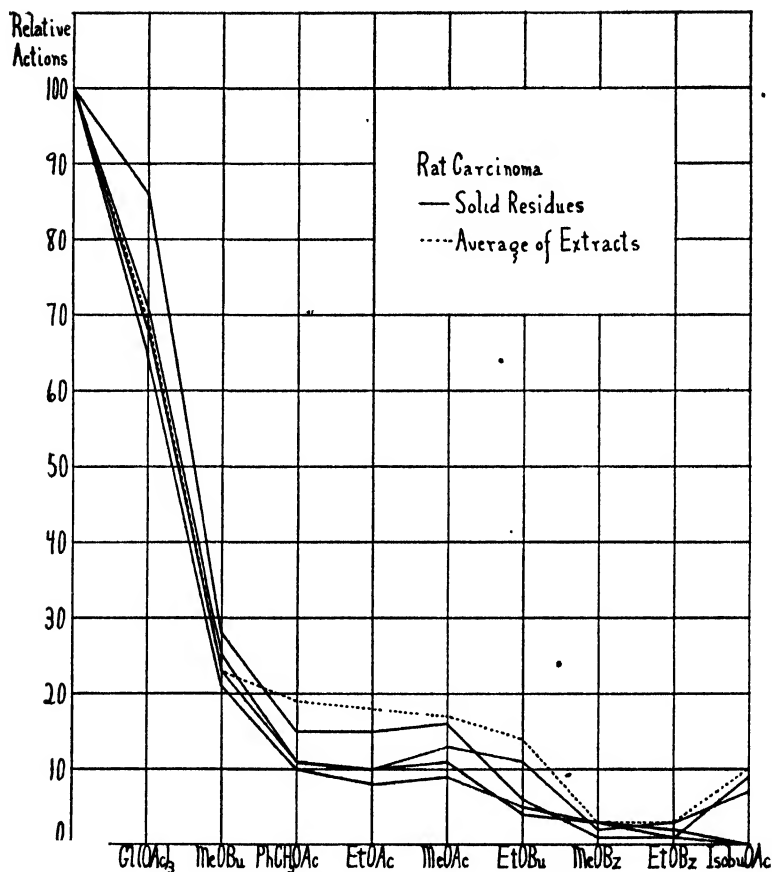


FIG. 2. Carcinoma. Solid residues. Since it is conceivable that the actions of extracts of tumors or tissues on esters may be different from those of the whole tumors or tissues, the solid residues after extraction were tested in a number of cases. The curves for the carcinoma residues show essentially the same pictures as the extracts. Because of the necessity of weighing the moist residues with the accompanying inaccuracy, the relative actions showed in some cases greater variations than did the extracts. In general, it may be said that for all the materials studied, the solid residues gave essentially the same types of relative actions as did the corresponding extracts. This eliminates the possibility that the relations observed are due mainly to solubility differences of the different ester-hydrolyzing enzymes.

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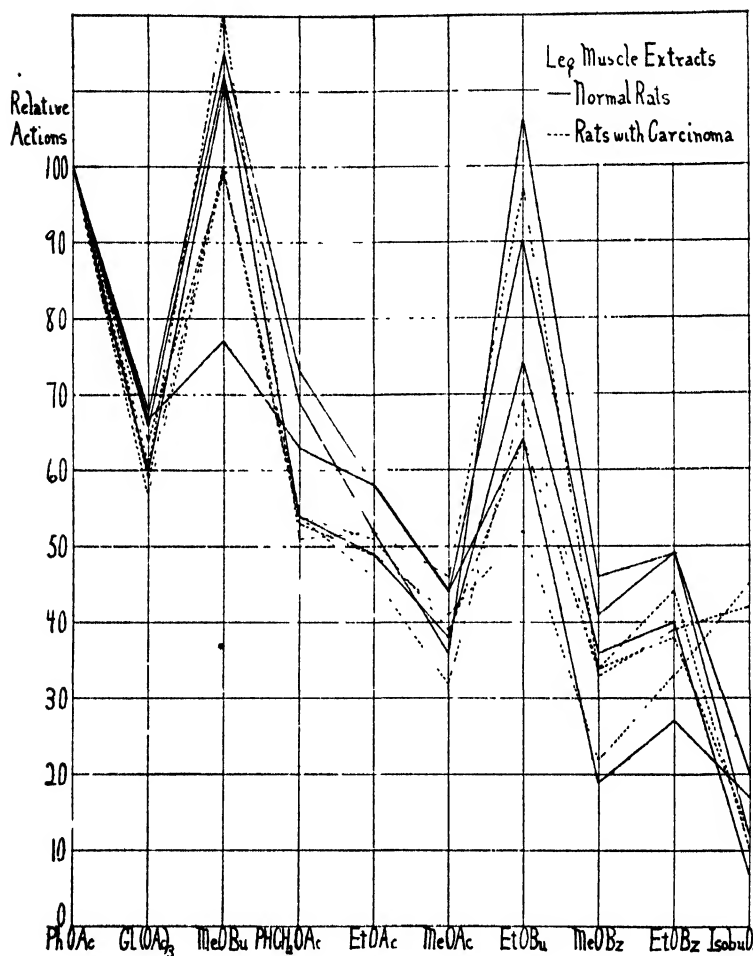


FIG. 3. Leg muscle extracts (normal and tumor-bearing rats). An entirely different picture from that of the tumor is shown by the leg muscle extracts. Some of the differences in the ethyl butyrate and isobutyl acetate results are due to the different samples of ester used. There are greater irregularities in the different curves of relative actions because of the smaller absolute actions and consequent greater influence of the experimental errors. The butyrates gave results as high as, or higher than, PhOAc. PhCH₂OAc actions were larger than EtOAc and MeOAc (true for no other rat tissue studied). The presence of tumors in the rats exerted no influence on the actions.

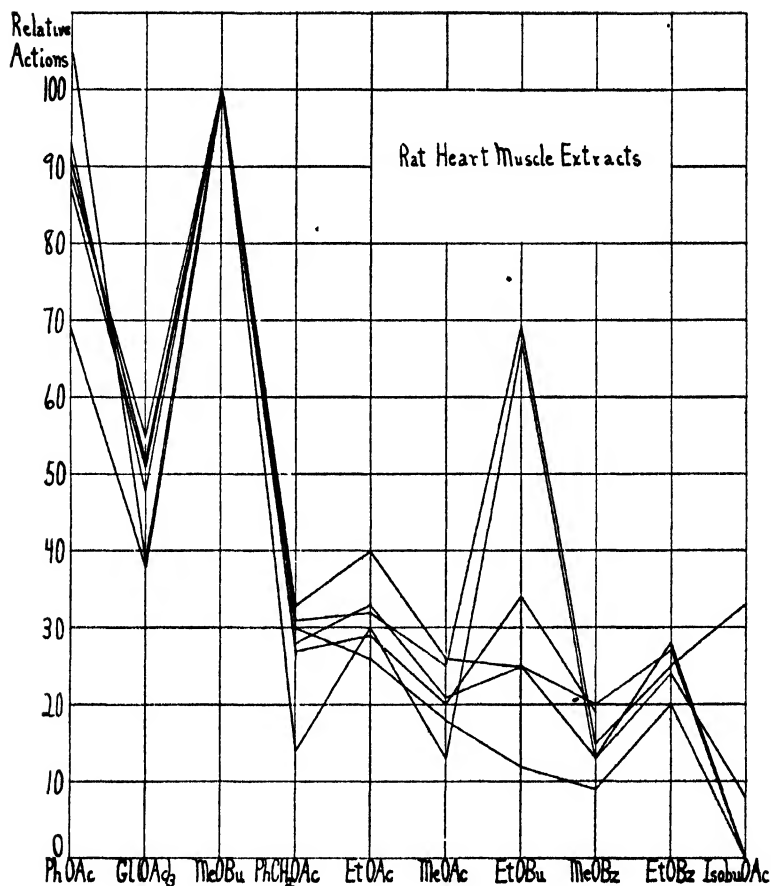


FIG. 4. Heart muscle extracts. The small absolute actions due to small amounts of material cause greater irregularities in the curves. The results differ from those of the leg muscle extracts in the smaller values for $\text{Gl}(\text{OAc})_2$, PhCH_2OAc , EtOAc , and MeOAc .

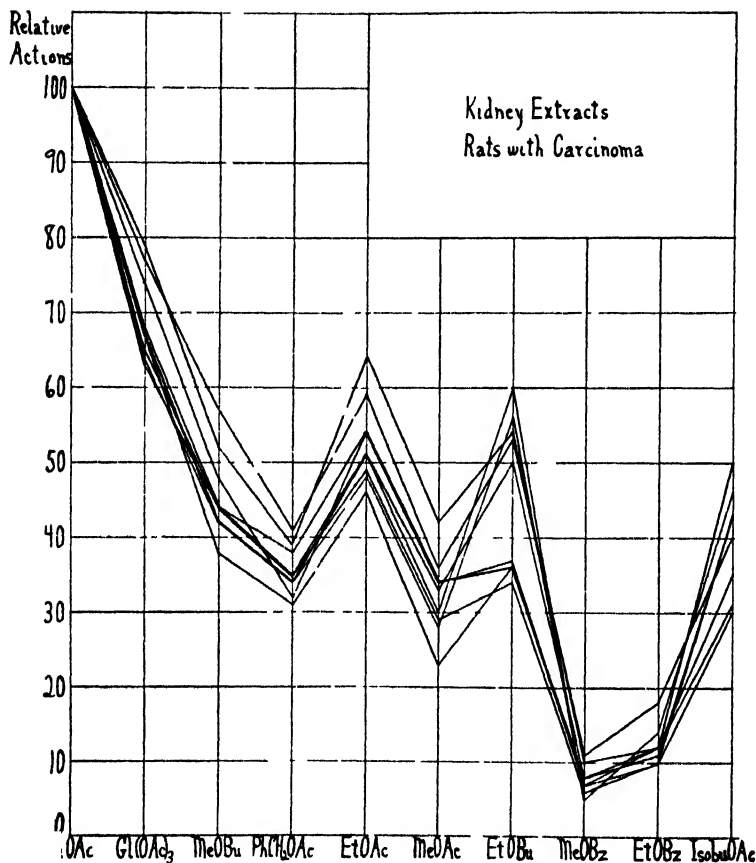


FIG. 5. Kidney extracts (tumor-bearing rats). Actions for ethyl esters as high as, or higher than, for methyl esters; while the acetates, aside from PhOAc, were as large as the corresponding butyrates.

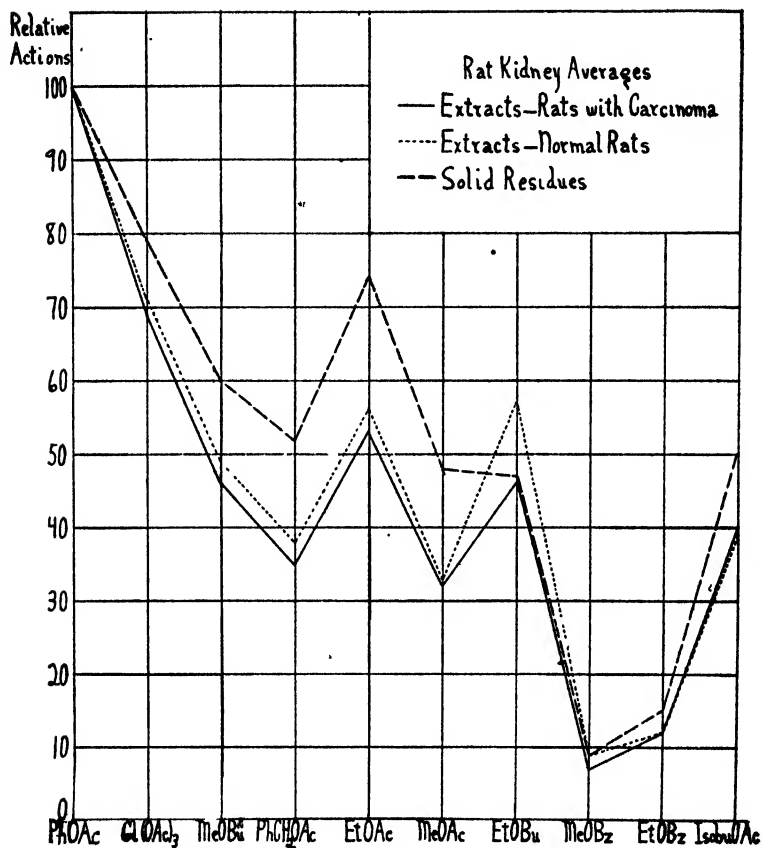


FIG. 6. Kidney averages (normal and tumor-bearing rats and solid residues). The averages show that the extracts of normal rat kidneys, of kidneys of tumor-bearing rats, and of the residues after extraction gave essentially the same results. As usual, the results with the residues showed greater irregularities.

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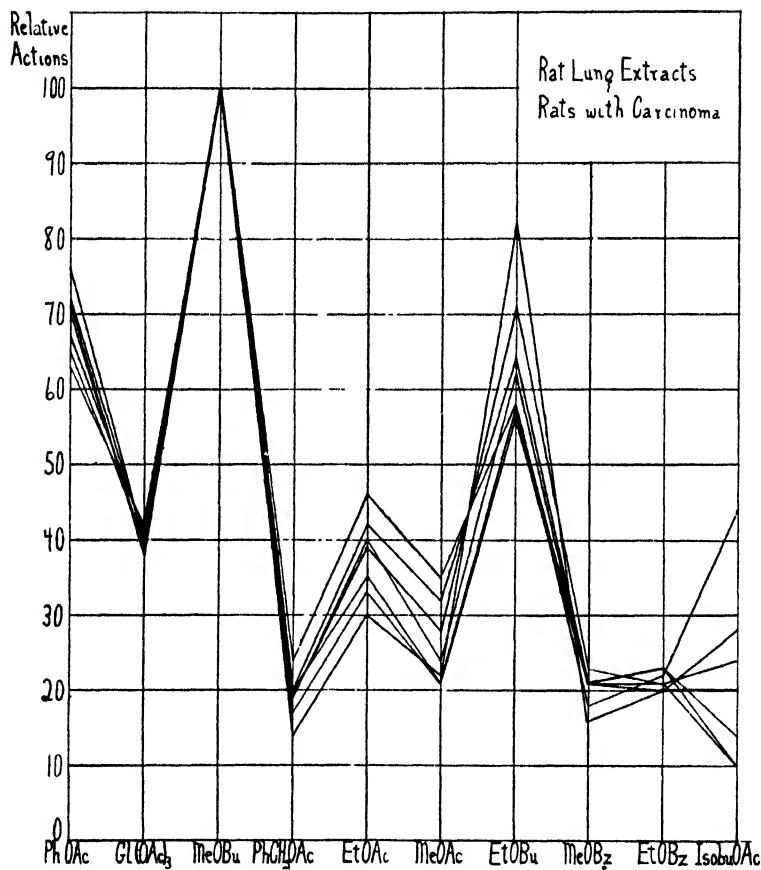


FIG. 7. Lung extracts (tumor-bearing rats). The large actions on MeOBu are striking. As for the rest, while the general picture is different, it is hardly necessary to call attention to the details.

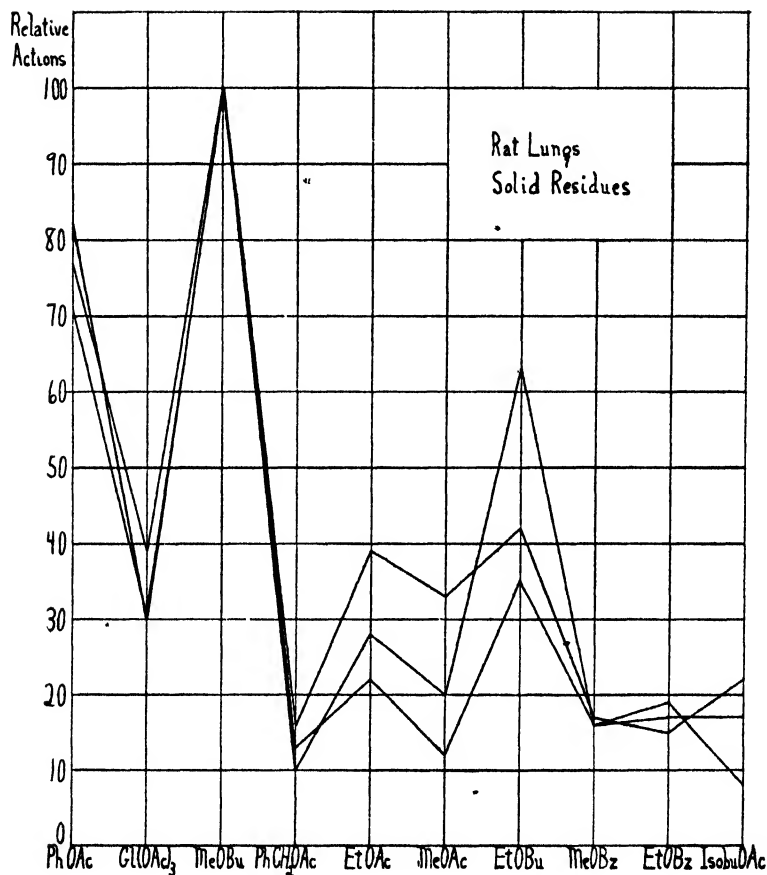


FIG. 8. Lungs. Solid residues. The picture is very similar to that of the extracts, with the usual greater irregularities.

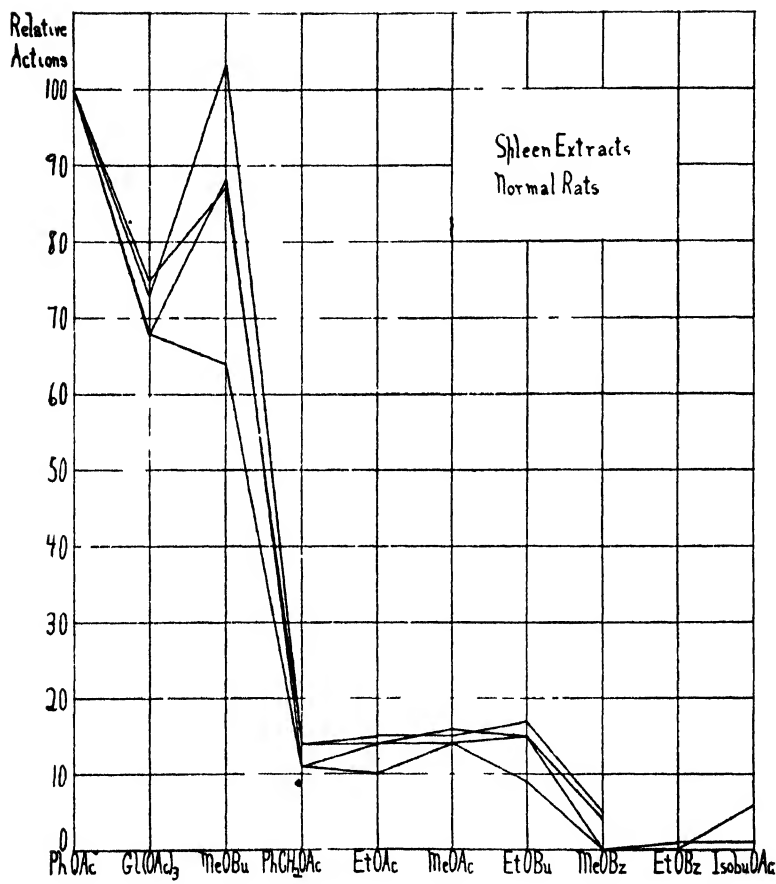


FIG. 9. Spleen extracts (normal rats).

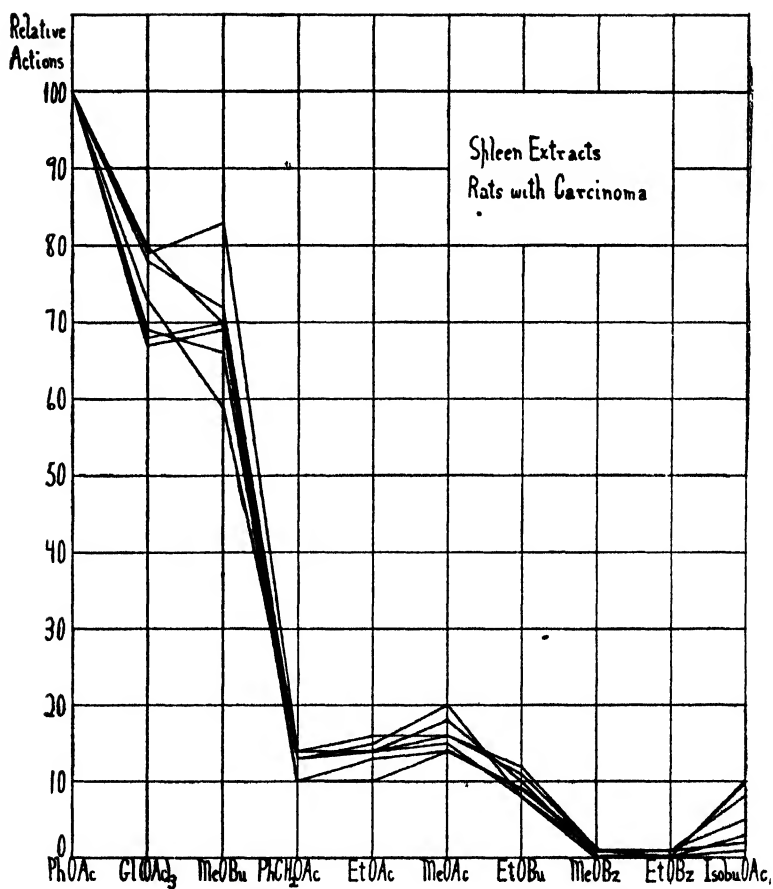


FIG. 10. Spleen extracts (tumor-bearing rats).

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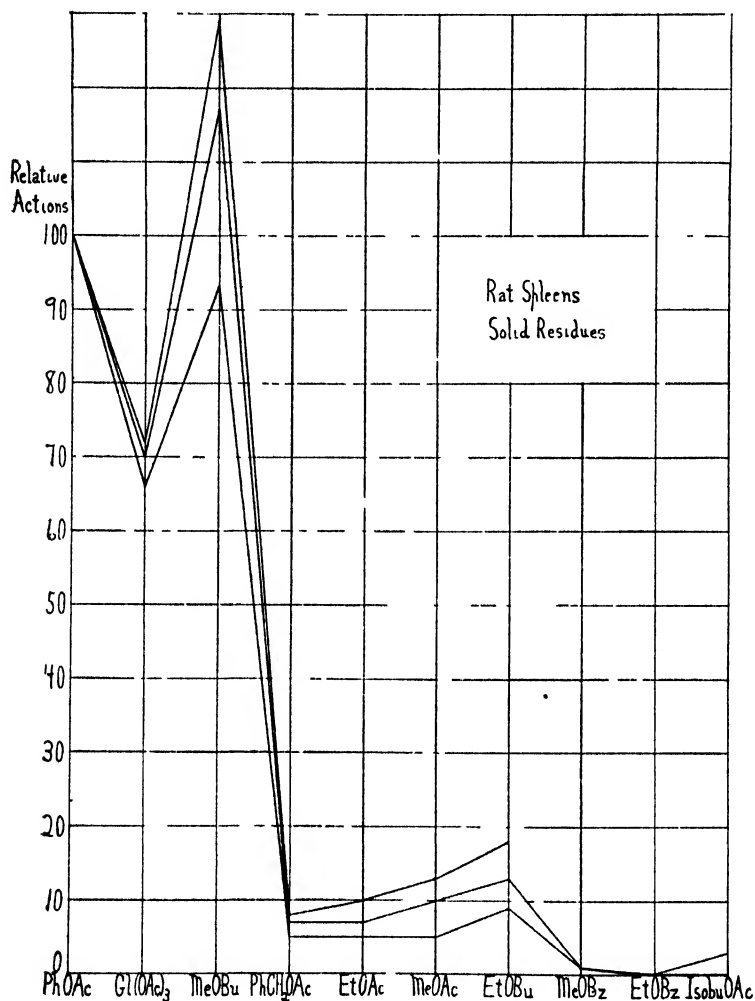


FIG. 11. Spleen. Solid residues. Figs. 9, 10, and 11 may be considered together. The pictures are much the same and similar to the tumor picture except for the methyl butyrate actions. These are somewhat irregular in all three charts. This may point to possible solubility differences for the different ester-hydrolyzing enzymes in the spleen. However, it is due more probably to a greater sensitiveness to external accidental influences of the methyl butyrate hydrolysis reaction. Similar influences, although much

smaller in magnitude, were observed in some of the actions of other tissues. Although the methyl butyrate actions were somewhat irregular, still the action was in every case 60 or more (as compared with phenyl acetate 100) so that there is no possibility of confusion with the tumor picture. Also, although the actions for the remaining esters show relations similar to those with the tumor, still a minor difference is observable in that the spleen actions on the whole are smaller (in comparison with the first three esters) than the tumor actions.

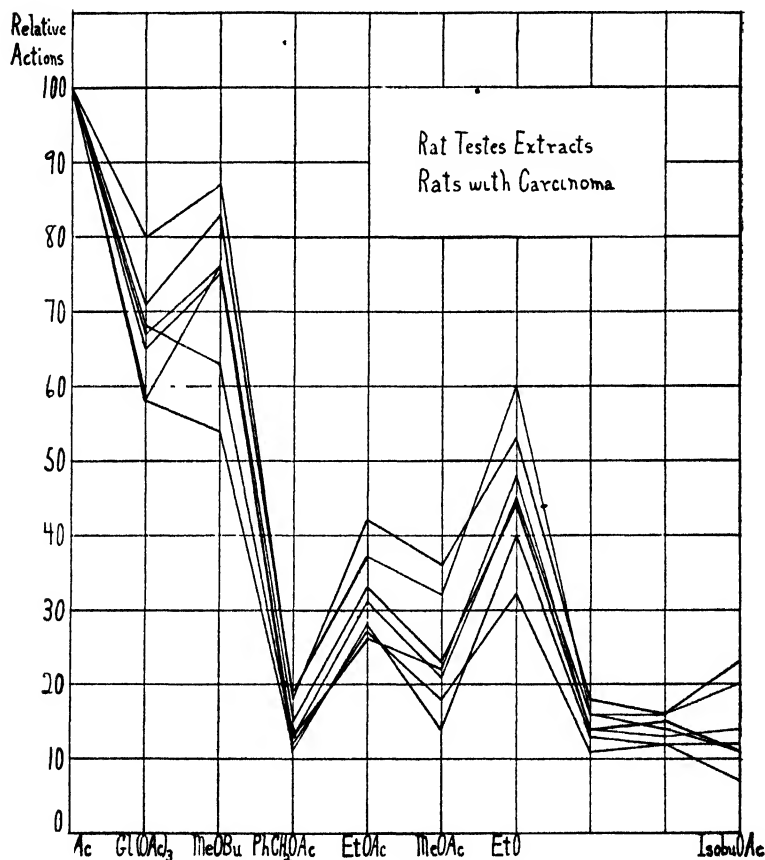


FIG. 12. Testes extracts (tumor-bearing rats).

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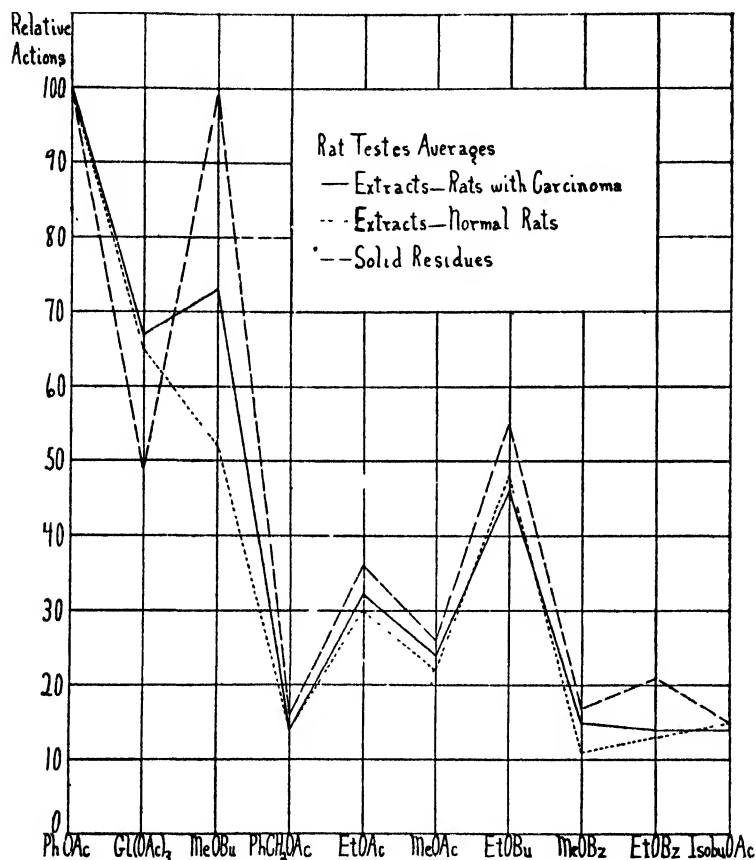


FIG. 13. Testes averages (normal and tumor-bearing rats and solid residues). The testes actions on methyl butyrate appear to be quite irregular, while those on glyceryl triacetate somewhat less so. However, if one of the other esters, not phenylacetate, were used as the standard, much of the irregularity would disappear except for the phenyl acetate results. This is true especially for Fig. 12. The solid residues, as usual, showed greater irregularities than did the extracts. It may be noted that the butyrate actions were greater than the corresponding acetate actions. While the testes results are not as clean-cut as those of some of the other tissues, still, in comparing the different charts, the testes "picture" is fairly characteristic.

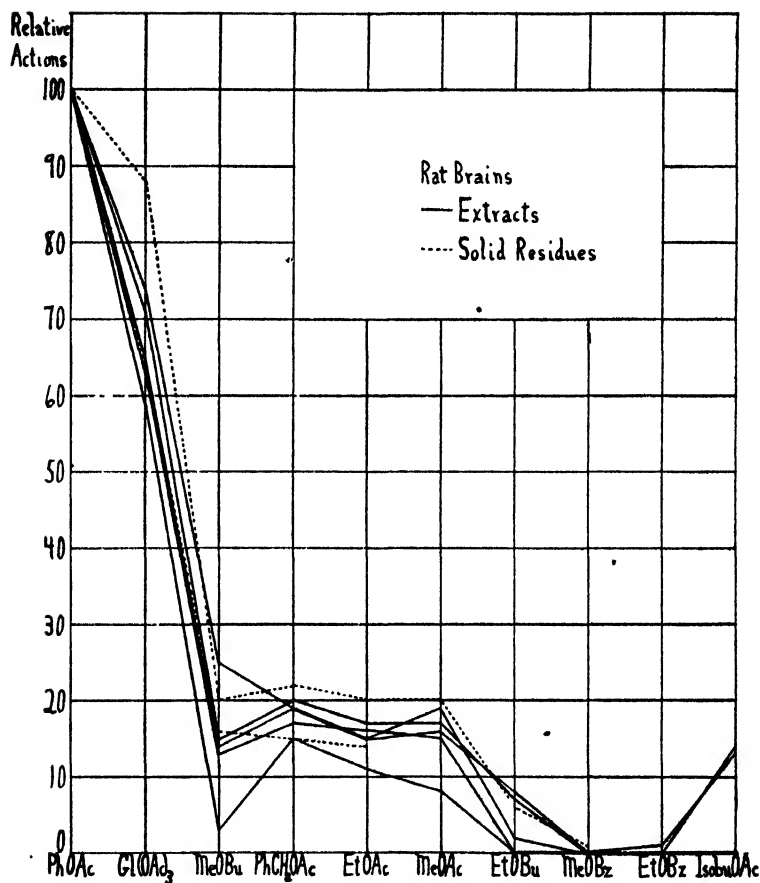


FIG. 14. Brain. Extracts and solid residues. These curves show greater similarities to the tumor curves than any other tissue. The absolute magnitudes of the actions as shown in Table I are not very different either, although somewhat smaller as a rule. The differences between the two pictures are of minor character and consist essentially in the smaller relative brain actions on the esters aside from methyl butyrate, and the fact that very little or no action was observed on ethyl butyrate and the benzoates. Also, it may be pointed out that with the isomeric esters, ethyl butyrate and isobutyl acetate, this is the only rat tissue in which the action was found to be markedly larger on the isobutyl acetate.

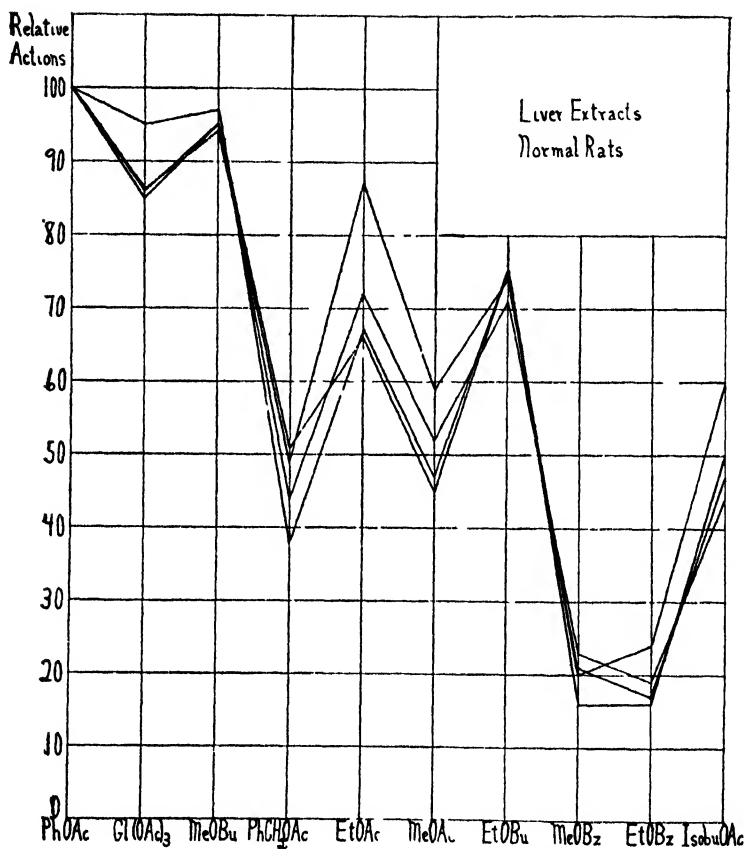


FIG 15 Liver extracts (normal rats).



FIG. 16. Liver extracts (tumor-bearing rats). The curves for the extracts of normal rat livers (Fig. 15) show more general enzyme actions than do those with any of the other tissues. The results with the liver extracts of the tumor-bearing rats (Fig. 16) show distinctly different relations although there is an underlying similarity between the curves. In several of the experiments secondary tumors were found in the livers and removed as far as possible. It is probable that they were present in other cases, especially in the earlier experiments, and not noticed since no microscopic examinations were made. The curves in Fig. 16, as a matter of fact, differ from those in Fig. 15 in that they begin to approach the characteristic tumor type (Fig. 1).

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! Emphasis has been placed so far more on the general pictures of the results than on any specific comparisons except in isolated cases. Some more specific relations will now be given.

Comparing the corresponding methyl and ethyl esters, it is seen that for the butyrates, the actions are greater in every case for the methyl ester than for the ethyl ester, except for the kidney where they are the same. For the acetates, the actions are less in every case for the methyl ester than for the ethyl ester except for the tumor, spleen, and brains, where they are the same. For the benzoates, the actions are less for the methyl ester with leg muscle, heart muscle, and kidney, and very nearly the same for the rest. The actions on methyl butyrate are greater in every case than on methyl acetate; greater for ethyl butyrate than for ethyl acetate with leg muscle, heart muscle, lungs, and testes; less with brains; and much the same with tumor, kidney, spleen, and liver.

In view of previous studies on glyceryl triacetate and ethyl butyrate (13, 14), a comparison of the former with the butyrates may be of interest. Comparing glyceryl triacetate and methyl butyrate greater action was found for the latter with leg muscle, heart muscle, lung, spleen, and testes; comparing glyceryl triacetate and ethyl butyrate, greater action was found for the latter with leg muscle, heart muscle, and lung.

The isomeric esters might also be compared. For example, greater action was observed on phenyl acetate than on methyl benzoate in every case, but the ratio varied from 100:50 for leg muscle to 100:0 for spleen and brains. With benzyl acetate and ethyl benzoate, very nearly the same actions were observed with heart muscle, lungs, and testes, while benzyl acetate was higher with the rest. Isobutyl acetate gave larger actions than ethyl butyrate with brains, the reverse was true for the other materials.

It is obvious that the curves show characteristic relations or types for the tumor and each tissue. These relations as given hold only for the conditions of the experiments which were used, but it would naturally be possible to obtain analogous curves or types for other conditions. In addition to the general "pictures" a more detailed study of the results, taken in pairs of esters or groups of esters, brings out relations, a few of which were given, which make it possible to characterize definitely the action of a

given tissue, or of a given extract as obtained by extraction of a tissue, perhaps unknown. In such comparisons, however, care must be taken not to assume definite relative actions for tissues and esters in cases where the absolute actions are small and introduce the possibility of error which might obscure the real relations. Reference to the results in Table I will show where the possibility of such errors might exist.

This raises the question of the absolute actions which were determined and which must necessarily be included for a proper understanding of the relations. It is advisable to compare extracts of the same amounts of original tumor or tissues. Even so, since different groups of rats were used, the absolute actions were found to differ considerably at times. It was only possible to obtain complete series for all the tissues in a few cases, and it might be misleading to present these results as conclusive evidence of such absolute action. It is preferable to state some general relations for the present.

The first striking fact which may be mentioned is the small value of the carcinoma action on all the esters. The heart and leg muscle and the brain actions are of the same order of magnitude, but the actions of the other tissues are very much greater. While there are differences which depend upon the ester used, it may be said that in general the actions were largest with the kidney and liver (not very different for the two), that the testes followed closely, and then the spleen and the lungs. While this statement is a very rough approximation to the facts, it may be pointed out that the order may be different in certain cases. Thus, the kidney is low with methyl butyrate while the lung and testes are high, etc. Such facts, however, do not invalidate the general statement of the relations.

For the sake of completeness, some of the results obtained for the protease actions of the tumor and tissue extracts are given in Table II. Only those for which the concentration corresponded to 8.9 mg. of tissue extracted per cc. of solution tested are given.

Considerable differences in the actions are evident. For the peptone, the order of decreasing magnitude of the actions is kidney, lungs, liver, spleen, testes, tumor, brains, and leg muscle. For the casein, the order is kidney, spleen, lungs, testes, liver and tumor, brains, and leg muscle. The small absolute values of the

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carcinoma actions are striking again, as well as the high value of the kidney. Also, the carcinoma showed greater action than the leg muscle, confirming the results already published (3). The order of the actions is obviously different for the two protein preparations.

The relative actions on the two preparations cannot be compared as readily as with the ester-hydrolyzing actions, since only two substrates were used, and also because a number of the actions, especially on the casein, were too small to use without introducing considerable errors into the relative actions. Even

TABLE II.
Protease Actions (Formol Method) of the Rat Carcinoma and Tissue Extracts.

Experiment No.	Tumor or tissue.	Actions in tenths of milli-equivalents.	
		Peptone.	Casein.
96	Carcinoma.	0.65	0.28
97	"	0.77	0.17
95	Leg muscle.	0.26	0.13
95	Kidney.	2.40	2.31
100 RT	"	2.39	2.27
95	Lung.	1.32	0.68
100 RT	"	1.20	0.41
95	Spleen.	0.92	0.74
100 RT	"	0.95	0.51
95	Testes.	0.90	0.47
95	Brains.	0.49	0.15
95	Liver.	1.12	0.29
97 RT	"	1.27	0.37

so, it may be pointed out that the ratio of the two actions for the kidney was very nearly unity, and that the relative action on the peptone became greater in approximately the order spleen, testes, lung, leg muscle, tumor, brain, and liver. For the liver the ratio of the actions was nearly 4 to 1.

It is evident that with a number of different protein substrates, series of results with different tissues would be obtained, which, when compared in the same way as the ester-hydrolyzing actions, would show analogous results. The few results given here for the protease actions are so similar to the lipase actions which were given in greater detail, that it is perhaps permissible to consider

that these lipase results are of deeper significance as regards the general enzyme characters of the materials than might be expected at first sight.

DISCUSSION.

The results which have been presented show definite characteristic "pictures" for the ester-hydrolyzing actions of the rat tumor and tissues. The protease actions were not studied in as great detail, but enough has been presented with these to show that analogous "pictures" might be developed with a number of protein preparations to make the same sort of differentiation possible. The relative actions of the various enzyme materials proved to be of the most significance in the interpretation of the results, but the absolute actions should also be considered. They aid in the study of the relative actions and also, in some cases, bring out interesting relations which might otherwise be ignored.

Several facts must be emphasized in the discussion of the results. In the first place, the curves presented and the relations developed refer to one special set of experimental conditions. While such curves and relations undoubtedly are typical of all studies of this nature, the details of the relations developed under different experimental conditions would unquestionably be quite different.

As for the actual results obtained, it is hardly necessary to add much to what was said in the preceding section. One point may however be referred to again. The small absolute values for the enzyme actions of the tumor, in comparison with the enzyme actions of a number of tissues are striking. These small actions will appear again in later papers dealing with tumors of human origin. Apparently, the magnitudes of the enzyme actions are not characteristic of tumors or "abnormal" growths, but rather certain characteristic types of action as exemplified by the Flexner-Jobling rat carcinoma actions.

The writers wish to thank Mr. Isaac Lorberblatt for his aid in carrying out the experiments described in this and the following papers.

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SUMMARY.

The ester-hydrolyzing actions on ten esters and the protease actions on two protein preparations of the Flexner-Jobling rat carcinoma and eight rat tissues were determined. The results are presented in the form of tables, for the absolute actions, and curves, for the relative actions. The tumor and each tissue present certain characteristic relations, especially with the ester-hydrolyzing actions, which may be considered typical of that material for the definite experimental conditions used.

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STUDIES ON ENZYME ACTION.

XXVI. COMPARATIVE LIPASE AND PROTEASE ACTIONS OF DIFFERENT BEEF TISSUES.

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INTRODUCTION.

In the preceding paper, lipase and protease actions of the Flexner-Jobling rat carcinoma and a number of rat tissues were described in some detail. It was shown that each tissue caused definite relative actions on a number of different substrates which could be plotted so that a characteristic "picture" of the enzyme actions of that tissue under the fixed conditions used, was obtained. In this paper, a similar study will be presented of a number of beef (steer) tissues. It was desired to determine whether, and to what extent, the corresponding tissues of different animals might be expected to show the same enzyme "pictures."

Experimental Methods and Results.

The experimental methods were in all particulars practically identical with those given in the preceding paper and therefore need not be repeated.

The results will be presented similarly. For the ester-hydrolyzing actions: first, a number of the absolute actions as determined experimentally will be given in Table I in order to show the magnitudes of the various actions and the probable degrees of accuracy; and second, the relative actions, as percentages of the greatest action in each series, in the form of graphs in Figs. 1 to 6.

TABLE I.

Hydrolyzing Actions in Tenths of Milli-Equivalents of Acid Produced by the Tissues on the Indicated Esters.

Experiment No.	Tissue extracted per cc. mixture tested.	PhOAc	Cl(OAc) ₂	MeOBu	PhCHOAc	EtOAc	MeOAc	EtOBu	MeOBs	EtOBs	IsobuOAc
Beef brain extracts.											
M 13	88.9	1.31	0.99	0.35	0.42	0.37	0.43	0.19	0.03	0.03	0.39
M 3	143.1	1.59	1.33	0.36	0.44	0.43	0.59	0.05	0.00	0.01	0.25
Beef brain residue after extraction.											
M 3	133.3	4.56	2.24	0.90	0.63	0.59	0.69	0.57	0.04	0.10	0.52
Beef kidney extracts.											
R 31	9.0	1.14	1.47	0.50	0.81	0.50	0.44	0.31	0.04	0.01	0.77
M 15	44.5	3.48	3.92	0.98	1.80	1.59	1.56	1.00	0.25	0.14	1.77
M 6	96.7	4.61	4.95	(3.30)	2.45	2.29	2.21	2.34	0.70	0.51	2.86
Beef spleen extracts.											
M 11	8.9	2.83	0.77	0.50	0.13	0.17	0.13	0.28	0.01	0.00	0.12
M 12	44.4	4.19	2.18	1.05	0.67	0.57	0.70	0.58	0.09	0.10	0.75
M 12	88.9	5.91	3.91	1.77	1.21	0.91	1.19	0.91	0.06	0.06	1.21
Beef spleen residue after extraction.											
M 5	33.3	4.20	3.04	1.46	0.50	0.46	0.53	0.71	0.06	0.00	0.33
Beef liver extracts.											
M 7	8.9	4.22	5.26	4.59	2.91	3.12	3.43	3.50	0.70	0.56	3.31
M 4	54.0	6.88	9.54	7.01	3.88	6.51	7.63	5.58	1.13	1.04	4.88
M 3	104.1	8.19	12.11	9.39	5.84	9.05	9.77	7.29	2.00	2.09	6.82
Beef liver residue after extraction.											
M 3	33.3	7.26	9.38	6.78	3.76	5.55	7.23	5.26	0.77	1.01	4.97
Beef heart muscle extracts.											
M 4	177.0	2.47	2.13	0.61	0.91	0.66	0.85	0.16	0.00	0.00	0.75
M 3	252.2	3.30	3.05	0.59	0.80	0.75	0.65	0.27	0.03	0.00	0.84

TABLE 1—*Concluded.*

Experiment No.	Tissue extracted per cc. mixture tested.	PhOAc	Gl(OAc) ₃	MeOBu	PhCH ₂ OAc	EtOAc	MeOAc	EtOBu	MeOBs	EtOBs	IsobuOAc
Beef heart muscle residue after extraction.											
M 5	mγ. 133.0	2.99	2.18	0.70	0.59	0.54	0.68	0.30	0.02	0.09	0.64
Beef lung extracts.											
R 31	8.9	1.86	0.44	1.71	0.46	0.35	0.57	0.66	0.14	0.14	0.37
M 10	8.9	2.34	0.53	1.46	0.35	0.54	0.35	0.60	0.16	0.11	0.33
M 3	46.8	4.68	2.97	4.53	1.96	1.98	2.53	2.90	0.79	0.80	1.85
Beef lung residue after extraction.											
M 3	53.3	8.61	4.04	5.86	2.09	2.45	3.59	3.97	0.68	0.60	2.22

The averages of the corresponding rat tissues are shown in the figures as broken lines. None of the results for the actions of the residues after extraction is shown in the figures. Essentially the same results, with somewhat greater inaccuracies due to the experimental conditions, were obtained as with the extracts. They are therefore omitted in order to simplify the graphs somewhat.

The results shown in Table I and in Figs. 1 to 6 may be discussed together.

It is evident from the figures that the "pictures" for the actions of beef kidney, liver, and lung are quite characteristic. The kidney is characterized by its relatively high glyceryl triacetate action, low methyl butyrate, high isobutyl acetate, and in general, acetate values nearly the same as, or higher than, corresponding butyrate values; the liver, by high glyceryl triacetate and high methyl butyrate, as well as a general high hydrolyzing action on all the esters; the lung, by a very low glyceryl triacetate action, especially in the more dilute solutions, and butyrate values higher than the corresponding acetates.

It is interesting to note that the three other beef tissues show "pictures" more or less similar to the Flexner-Jobling rat carcinoma "picture." Minor differences in the relative actions may

be pointed out. Beef brain shows very small ethyl butyrate action; beef spleen, small butyrate and benzoate actions; beef

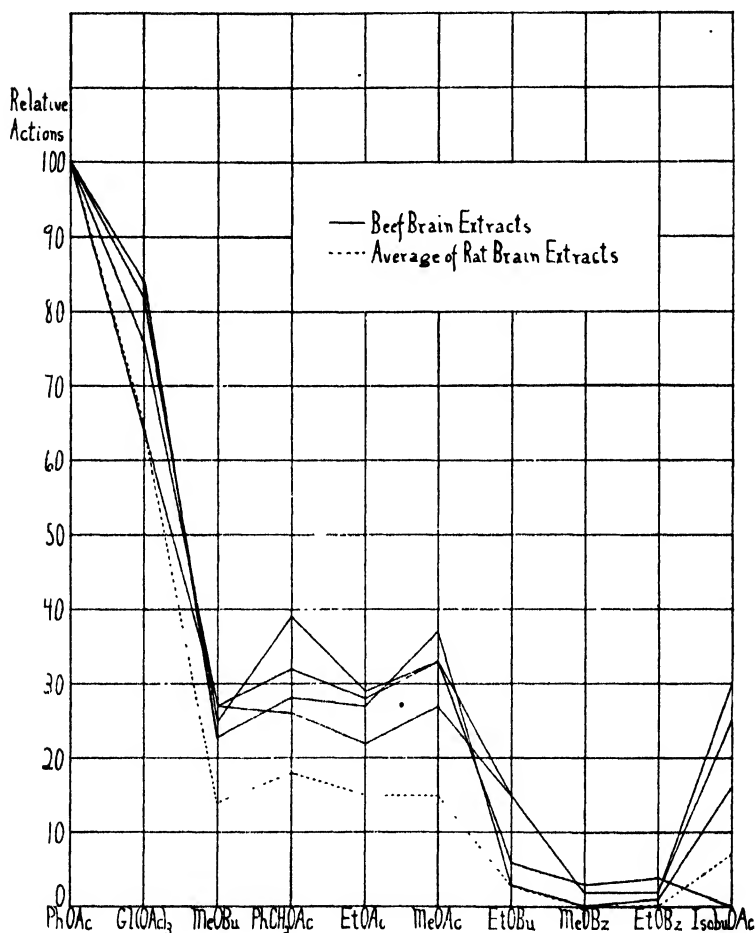


FIG. 1. Beef brain extracts. Rat brain average. In this and the following figures the ordinates show amounts of hydrolysis in terms of percentage action upon equivalent amounts of the indicated esters which are plotted at equidistant intervals on the abscissa axis.

heart muscle, very low butyrate values, and glyceryl triacetate values only slightly lower than phenyl acetate values. Real

differences are shown by the absolute actions as compared with the rat tumor. They are very much less for the beef brain and heart, somewhat less for the beef spleen.



FIG. 2. Beef kidney extracts. Rat kidney average.

Comparing the corresponding beef and rat tissues, it is seen that a strong similarity exists with the brains, that the spleens differ mainly in the action on methyl butyrate, that with the

kidneys, liver, and lungs certain similarities exist but are overshadowed by the differences, and that with the heart muscles no similarities are apparent, the differences being very striking.

The absolute actions of the beef tissues are very much less than those of the corresponding rat tissues except for the liver.

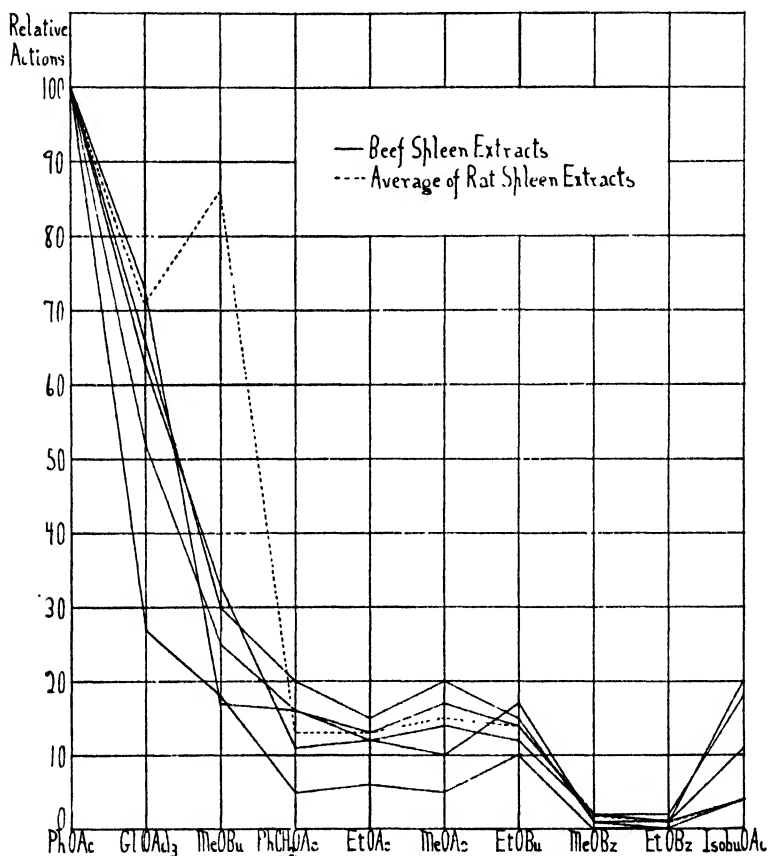


FIG. 3. Beef spleen extracts. Rat spleen average.

Although the types of the actions are different for the latter, the actions on the different esters are more nearly of the same order of magnitude, the beef liver showing greater action for some esters, the rat liver for others.

The comparative absolute actions of the various beef tissues on the esters are strikingly different from those of the rat tissues. Although a certain number of irregularities appear due mainly to the nature of the experiments, it is seen that the liver shows the

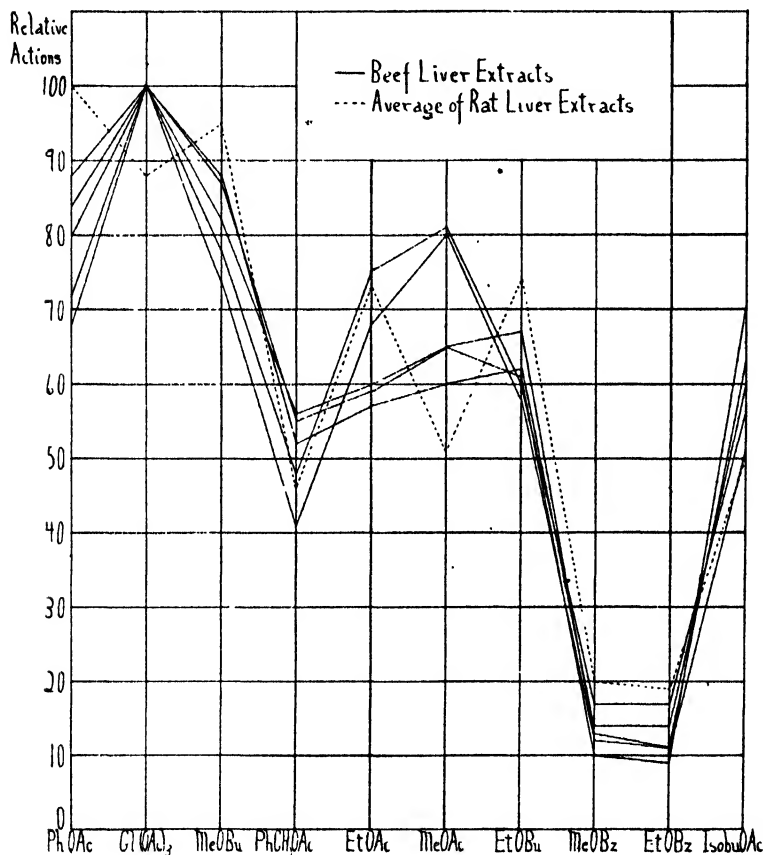


FIG. 4. Beef liver extracts. Rat liver average.

greatest action in every case. The order of the magnitudes of the actions with the remaining tissues are different for the different esters. It need only be mentioned that, for example, kidney is second with glyceryl triacetate, and lung with methyl butyrate.

It is hardly necessary to enter into further details with these relations. The heart muscle and the brain show the smallest actions in every case.

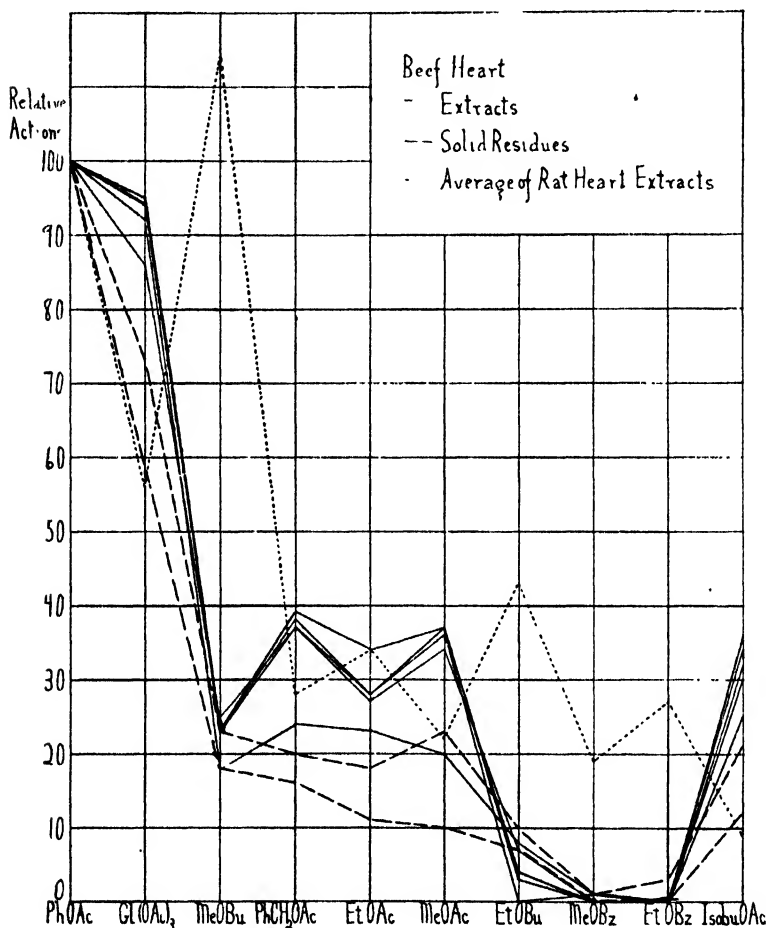


FIG. 5. Beef heart muscle extracts. Rat heart muscle average.

Some of the results for the protease actions of the beef tissue extracts are shown in Table II. The experimental methods were described in detail in the preceding paper.

The kidney extracts show the greatest actions on the two protein preparations, the liver next, followed by the lungs and spleens, with the brains showing the smallest actions. The ratios for the

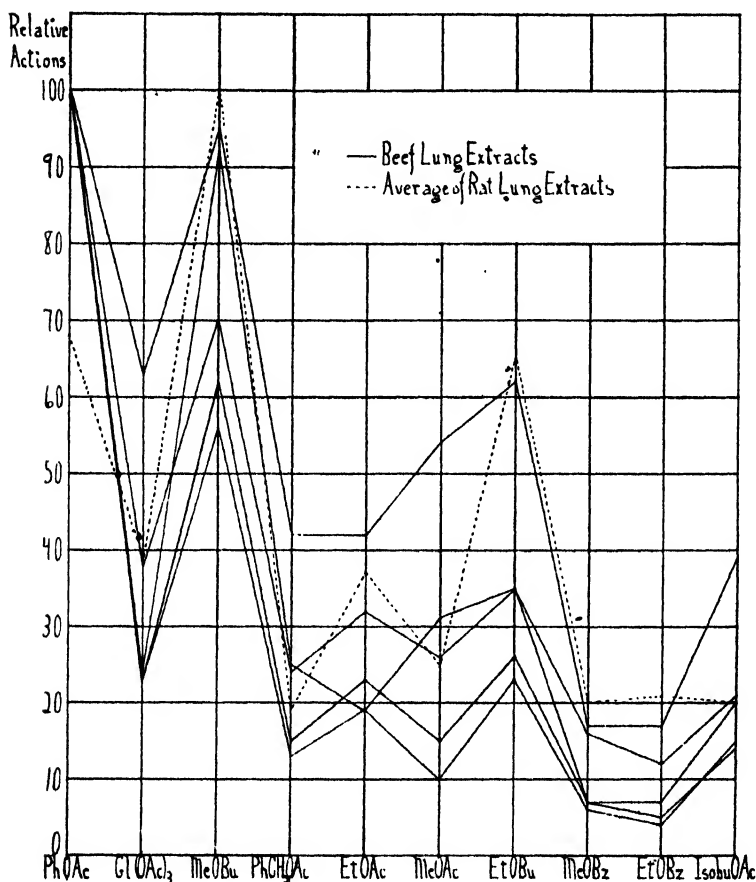


FIG. 6. Beef lung extracts. Rat lung average.

actions on the peptone and casein do not differ markedly for the brains, kidneys, spleens, and livers, but for the lungs the action on the casein is practically zero.

TABLE II.

Protease Actions (Formol Method) of Beef Tissue Extracts.

Experiment No.	Tissue tested.	Tissue extracted per cc. solution tested.	Actions in tenths of milli-equivalents.	
			Peptone.	Casein.
		<i>mg.</i>		
M 13	Brain.	8.9	0.08	0.06
M 13	"	88.9	0.82	0.29
R 31	Kidney.	9 0	1.70	0.55
M 15	"	17.8	2.18	0.70
M 15	"	44.5	2.56	0.72
M 6	"	96.7	2.43	1.04
M 11	Spleen.	8.9	0.98	0.27
M 12	"	8.9	0.72	0.30
M 12	"	44.4	1.35	0.54
M 11	"	88.9	1.44	0.40
M 7	Liver.	8.9	1.00	0.63
R 22	"	9 0	0.91	0.34
M 15	"	17.8	1.15	0.43
M 10	Lung.	8.9	0.82	0.05
M 10	"	44 5	1.73	0.09

DISCUSSION.

The results presented for the beef tissues show that it is possible to develop characteristic "pictures" for the various tissues here as well as for the rat tissues described in the preceding paper. While some of the "pictures" do not differ as strikingly as might be desired, it must be pointed out that only a limited number of esters was used. With a greater number and especially with esters of different types, greater differences unquestionably would be observed. The same relations hold for the protease actions of the tissues.

Comparing the corresponding tissues of the beef and the rat, it was pointed out that some show very much the same "pictures," others show small differences, while others show entirely different characteristics. Explanations for these similarities and differences might be advanced, but for the present, at any rate, it seems advisable to gather data for other animal tissues before developing such views.

SUMMARY.

The ester-hydrolyzing actions of six beef tissues on ten esters and the protease actions on two protein preparations were determined. The results are presented in the form of tables for the absolute actions, and curves for the relative actions. The characteristic relations for the various tissues are discussed and compared with the corresponding rat tissues.

STUDIES ON ENZYME ACTION.

XXVII. THE COMPARATIVE ENZYME ACTIONS OF TISSUE MIXTURES AND OF TUMOR-TISSUE MIXTURES IN RELATION TO THE COMPARATIVE ENZYME ACTIONS OF TISSUE AND OF TUMOR EXTRACTS ALONE.

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INTRODUCTION.

In discussing the characteristic "pictures" of the enzyme actions of the Flexner-Jobling rat carcinoma and of the tissues of the rat and other animals, as shown for example by their relative actions on a number of esters, it was suggested by Professor S. R. Benedict and others that the possible presence of certain substances peculiar to a given tissue might conceivably modify an enzyme behavior common to all tissues. In this way, the different pictures obtained for the various tissues would be accounted for without the necessity of assuming a characteristic enzyme behavior for each separate tissue. This explanation, if correct, would change the study from that of different, though related, enzymes in different tissues to a study of the actions of various substances on one enzyme or one group of enzymes. The view can be readily tested by studying mixtures of tissue extracts and of tumor and tissue extracts. The experimental results covering some of these studies will be presented in this paper.

Experimental Methods and Results.

The experimental methods were the same as those used in obtaining the results communicated in the preceding papers and

described in detail in "Studies on enzyme action. XXV" (1). The only additions to be made include the facts that in the mixtures of tissue extracts and of tumor-tissue extracts, 5 cc. of each extract were used and 5 cc. of water added to make up the usual 15 cc., and in preparing the boiled extracts, these were boiled over a free flame for 5 minutes, made up to original volume, and then 5 cc. portions diluted to 15 cc. with water, or mixed with 5 cc. of unboiled extract, 5 cc. of water added and tested in the usual way.

The results on the ester-hydrolyzing actions are presented in Figs. 1 to 6. They cover mixtures of beef kidney and beef lung; beef kidney and beef liver; and rat tumor with rat spleen, rat lung, rat kidney, and rat liver. The results with human tumors will be reserved for a later communication.

The results are plotted as absolute actions (tenths of milli-equivalents of esters hydrolyzed). The concentrations of tissue and tumor extracted corresponded to 8.9 to 9.0 mg. of each per cc. of final solution tested except for the rat tumor-rat liver mixture where the tumor concentration was doubled.

Fig. 1. In this and the following figures the ordinates show amounts of hydrolysis in tenths of milli-equivalents of acid formed from equivalent amounts of the indicated esters which are plotted at equidistant intervals on the abscissa axis.

Beef liver-beef kidney mixtures. The kidney and boiled liver-kidney actions were practically identical, as were also the liver and boiled kidney-liver actions. The liver-kidney mixture was, if anything, slightly less active than the boiled kidney-liver mixture, and definitely less active than the sum of the liver and kidney actions (upper curve). The types of actions (relative actions on the various esters) were very nearly the same for the "Found" and "Calculated" mixtures. These would be more obvious if the relative actions on the percentage scale had been plotted in place of the absolute actions as such. The fact that the "Calculated" actions were greater than the "Found" actions may be taken only as further evidence of the fact that the actions of even one tissue alone on the esters are not proportional directly to the concentration of that tissue in the extract as shown in a previous paper (2). The application to the results with mixtures of two different tissues follows from this relation.

The results of the mixture of the liver and kidney, because of a similarity of their relative actions separately on the different esters, are not so striking as those shown in Fig. 2.

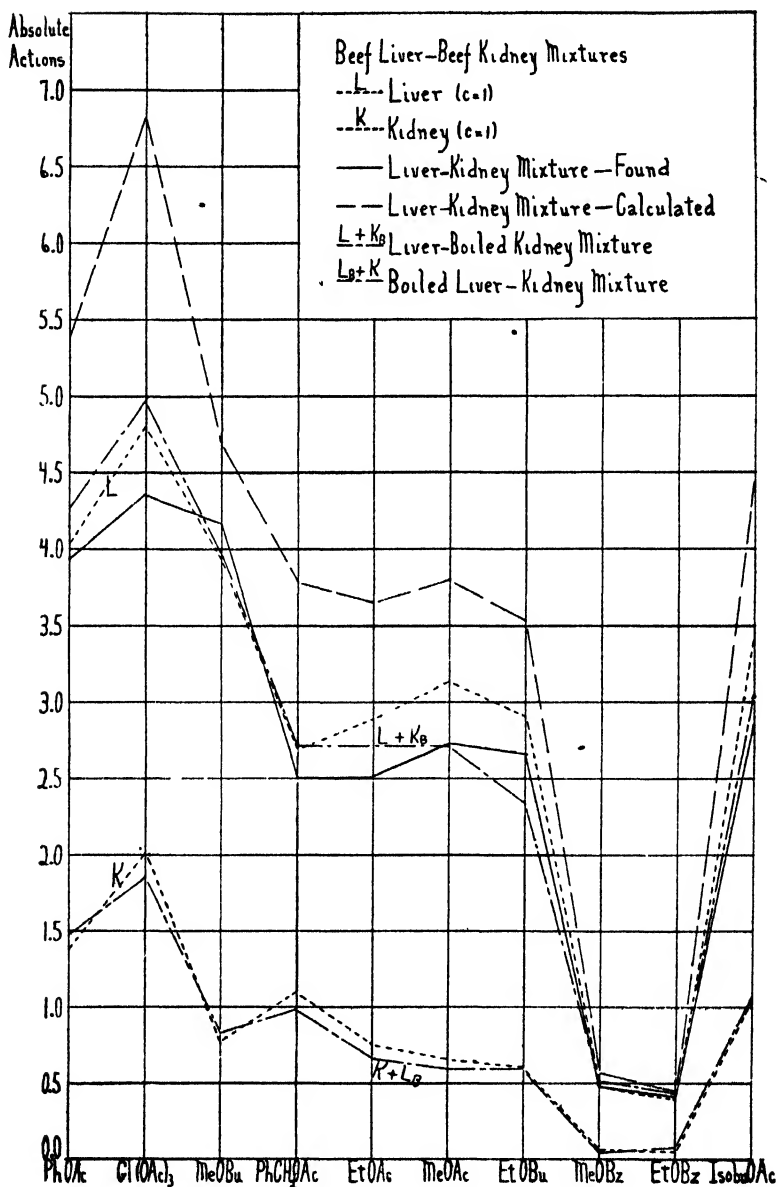


FIG. 1.

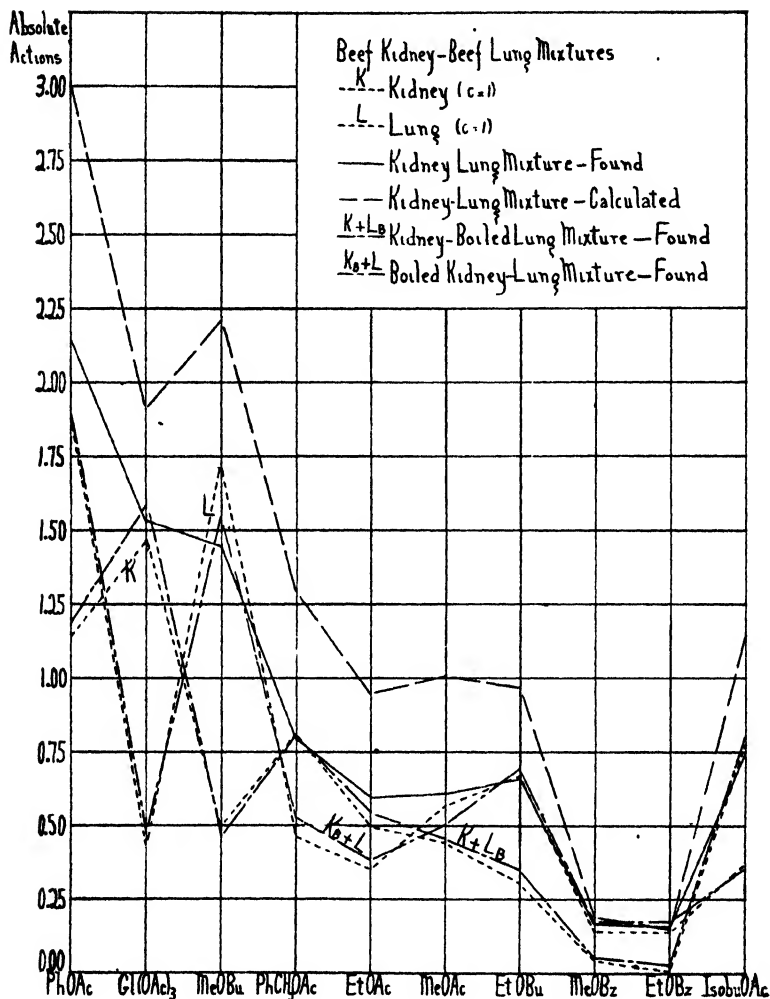


FIG. 2. Beef kidney-beef lung mixtures. The kidney and lung actions alone present entirely different pictures. Addition of boiled extracts did not affect the results in any way. The "Calculated" mixture gave absolute actions greater than the "Found" mixture, but the two pictures (relative actions or shapes of curves) are very nearly the same. The action of methyl butyrate (or perhaps glyceryl triacetate) only shows a small irregularity. If calculated in terms of percentages, the agreement between these relative actions would be still more apparent.

That the differences between the values for the "Found" and "Calculated" mixtures are due to concentration effects as stated may be shown by means of the results of Experiment M 15 in Table I.

Each solution was made up to 15 cc. and tested in the same way. Columns 2 and 3 in the table give the results obtained with the liver and kidney separately at a definite concentration for each; Column 4, the results for the mixtures of these same concentrations, the values being considerably less than the sums of the

TABLE I.
Ester-Hydrolyzing Actions of Beef Liver and Beef Kidney Extracts Alone and Mixed.

	Absolute actions found with tissue extracts.				Calculated. One-half the sum of 5 L and 5 K.
	5 cc. L*	5 cc. K	5 cc. L + 5 cc. K	2.5 cc. L + 2.5 cc. K	
PhOAc.....	2.92	1.00	3.15	2.09	1.96
Gl(OAc) ₃	3.65	1.76	3.74	2.53	2.71
MeOBu.....	2.80	0.21	2.90	2.09	1.51
PhCH ₂ OAc.....	1.87	0.91	2.11	1.35	1.39
EtOAc.....	1.98	0.66	1.88	1.31	1.32
MeOAc.....	2.01	0.61	2.07	1.32	1.31
EtOBu.....	2.26	0.34	2.11	1.34	1.30
MeOBz.....	0.44	0.04	0.52	0.27	0.24
EtOBz.....	0.38	0.00	0.47	0.25	0.19
IsobuOAc.....	1.99	0.84	2.37	1.58	1.42

*L represents liver; and K, kidney.

actions in Columns 2 and 3 except for the benzoates; Column 5, the results for the mixtures with the concentrations half those in Columns 2 and 3 (or in Column 4); and Column 6, the results calculated from the sums of the actions in Columns 2 and 3 and divided by 2. The results in the last two columns are comparable. They show striking agreements between the found and calculated results except for one ester, methyl butyrate. In other words, at similar concentrations for the mixture, the absolute actions as well as the relative actions of the mixture of tissue extracts are made up additively of the actions of the separate tissue extracts.

With the rat tumor and tissues, studies with boiled extracts were not carried out because of the small amounts of the materials available.

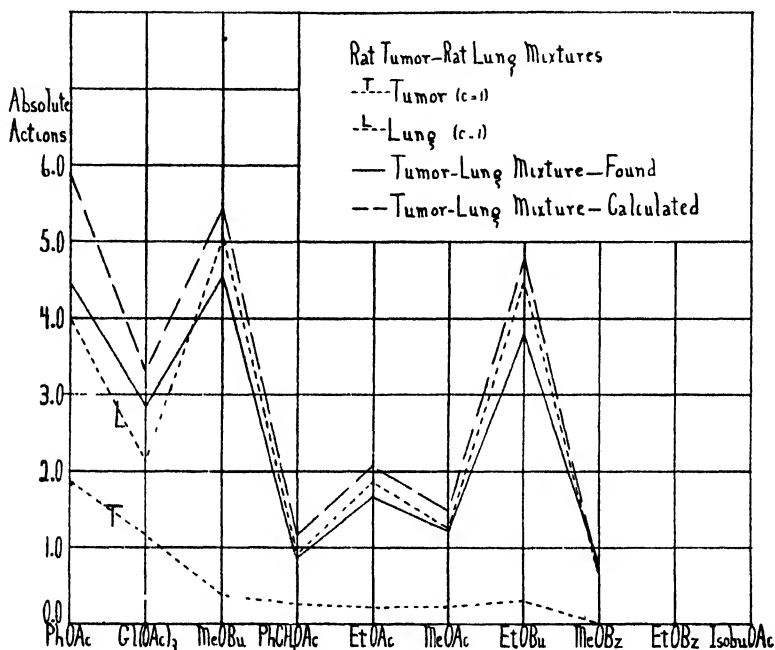


FIG. 3. Rat tumor-rat lung mixtures.

The results shown on Charts 3 to 6 may be considered together. They show clearly that the type or picture of the relative actions found in every mixture is essentially the same as that calculated from the separate actions. The absolute actions of the experimentally determined mixture are, however, again less in every case than the sums of the separate actions. This is not surprising, as already pointed out. It may be noted, on the other hand, first, that the absolute tumor actions in every case are much less than the absolute tissue actions; and second, the striking facts that the tumor-kidney mixtures show smaller absolute actions than the kidney alone. This is also true for some of the esters with the tumor-lung and tumor-spleen mixtures as compared

with the lung and spleen actions alone. For the tumor-liver mixtures, the actions throughout are greater than the actions of the liver alone. The differences are not large in any case, and they are not such as to change the characters of the curves or pictures of the "Found" mixtures as compared with the "Calculated" mixtures. It is, of course, impossible to state at

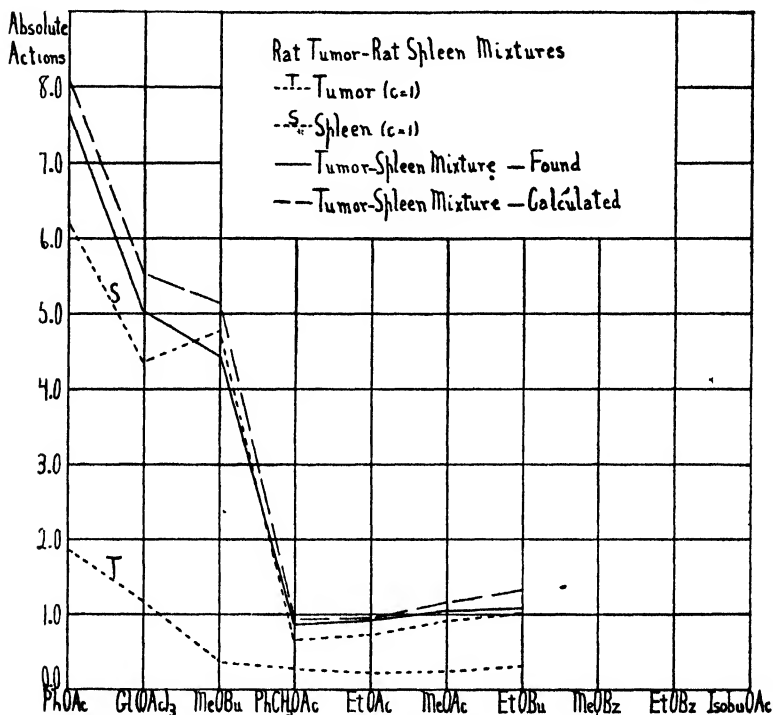


FIG. 4. Rat tumor-rat spleen mixtures.

the present time whether these small effects are produced by the tissue acting on the tumor or *vice versa*. They can only be noted as being of secondary significance in connection with the main problem under investigation.

Some results with protease actions are shown in Table II. The conditions of the experiments were the same as those stated in the paper on "Studies on enzyme action. XXV," plus those given in the earlier part of this paper.

The results in Table II need little additional explanation. The ratios of the actions on the two preparations show very good agreements between the found and calculated (from the separate deter-

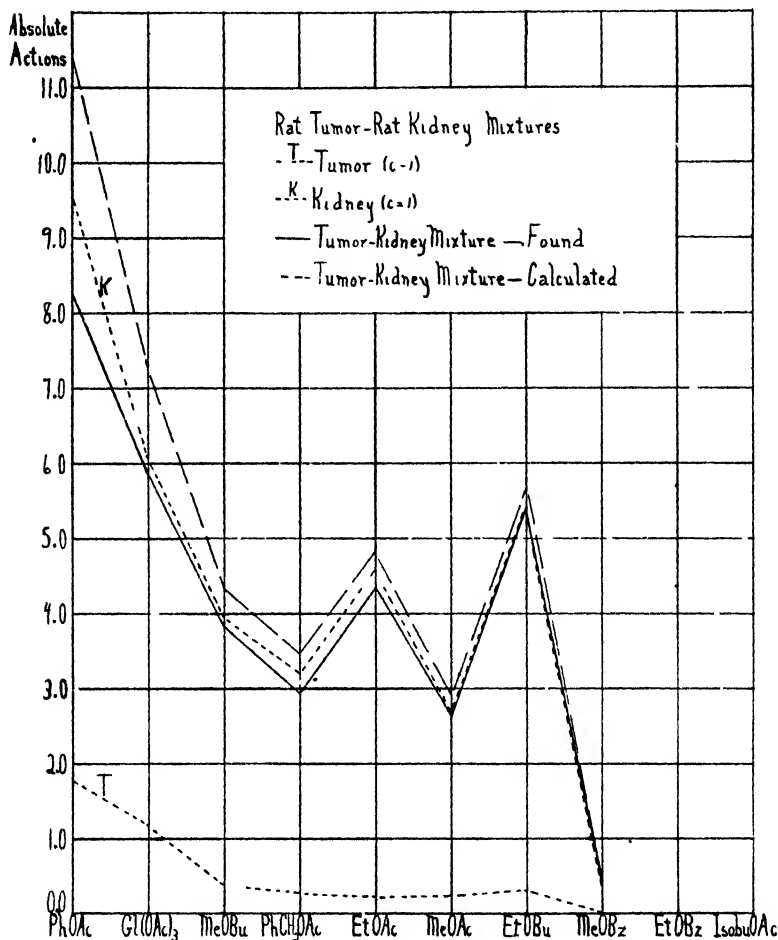


FIG. 5. Rat tumor-rat kidney mixtures.

minations) values in the first two experiments; not quite so good in the third, although in view of the magnitudes of the actions and the differences between the values of the ratios of the tumor and liver alone, no specific action of the one or the other can be said to

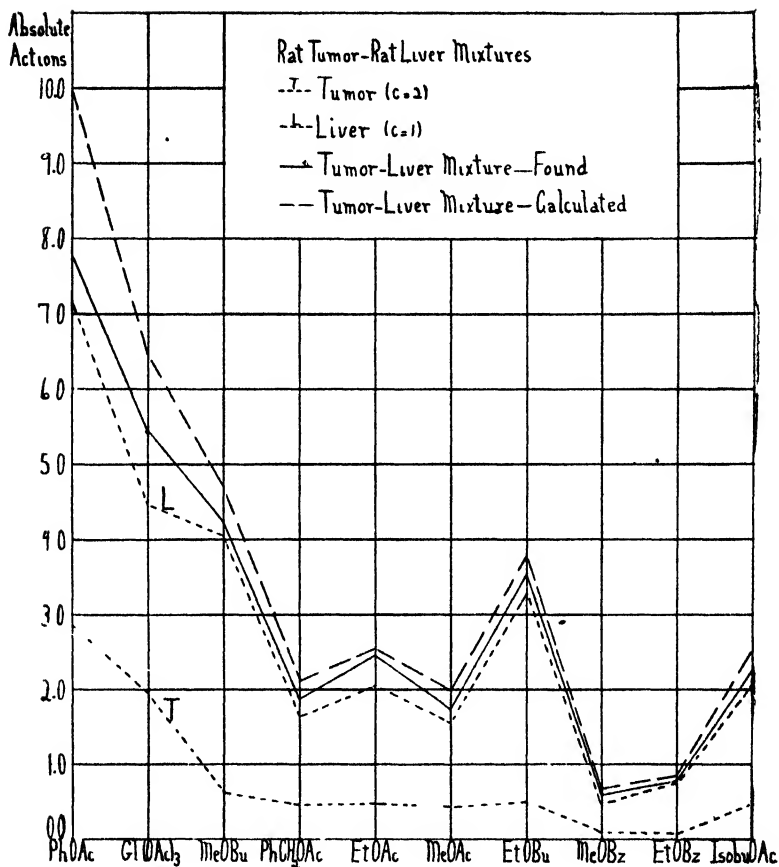


FIG. 6. Rat tumor-rat liver mixtures.

have occurred. The absolute actions found for the mixtures are, in every case, less than the actions calculated, as with the ester-hydrolyzing actions, and may be referred to similar causes.

TABLE II.

Protease Actions of Some Tissue and Tumor Extracts Alone and in Mixtures.

Experiment No.	Material tested.	Actions on:		Ratio of actions. Peptone: casein.
		Peptone.	Casein.	
R22 Beef.	Kidney.	1.69	0.48	3.52
	Liver.	0.91	0.34	2.68
	Kidney+liver, found.	1.93	0.60	3.22
	“ + “ calculated.	2.60	0.82	3.17
R31 Beef.	Kidney.	1.70	0.55	3.09
	Lung.	0.76	0.35	2.17
	Kidney+lung, found.	1.75	0.68	2.57
	“ + “ calculated.	2.46	0.90	2.73
100 Rat.	Tumor.	0.94	0.39	2.41
	Liver.	1.10	0.20	5.50
	Tumor+liver, found.	1.30	0.47	2.77
	“ + “ calculated.	2.04	0.59	3.46

DISCUSSION.

The results presented in this paper show that, for the mixtures and actions studied, no specific influence on the enzyme action which can be ascribed to a substance or substances present in a given tissue or tumor and perhaps peculiar to that material, was observed. The results obtained by Loevenhart (3) a number of years ago point in the same direction. He found that with extracts of the liver and pancreas of the dog and pig, the activity of mixtures for the lower fatty acid esters was made up only of the activity of the ingredients. With olive oil and pancreas extracts in mixtures, increases over those calculated for the separate extracts were found. These, however, may well have been due to other causes such as, possibly, solubility effects, etc.

The evidence presented here relates in the main to the relative actions on the various substrates for each series. The absolute actions of mixtures were found in every case to be less than the

sums of the actions of the constituents of the mixtures separately. As shown experimentally in one case, these differences may be referred mainly to the fact that the enzyme action of a given material in the case of those studied was not proportional to its concentration. Consequently it is not surprising that the action of a mixture containing two materials each of a definite concentration is less than the actions of the materials alone each of the same concentration as in the mixture. At the same time, certain minor influences of some of the tissue extracts on the absolute actions of the mixtures were observed which require confirmation and more extended study before anything definite can be said with reference to them.

CONCLUSION.

The enzyme actions of the Flexner-Jobling rat carcinoma and of a number of animal tissues were found to be peculiar to the tumor or tissues and independent of certain substances, distinct from the enzyme.

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THE EFFECT OF FASTING (AND REFEEDING) ON THE CALCIUM AND INORGANIC PHOSPHORUS IN BLOOD SERUMS OF NORMAL AND RACHITIC RATS.

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(Received for publication, December 21, 1923.)

McC'ollum and his coworkers (1) have shown that in rats with rickets a period of fasting induces a cessation of the activity of the disease and initiates healing with recalcification of the proliferative cartilage in the bones.

Kramer and Howland (2) have shown this calcification to be accompanied by changes in the inorganic salts of the blood, which changes are, as far as I am aware, concerned only with the calcium and inorganic phosphorus. These workers have further shown (3) that there is a close relationship between the deposition of calcium phosphate in bone and the product of the concentration of the serum calcium (in mg. per 100 cc.) into the concentration of inorganic phosphorus, expressed in the same terms. Their conclusion (3) is that "when the product is below 30, rickets is to be expected, between 30 and 40 it is probable. When the product is above 40 either healing is taking place or rickets is entirely absent." This is well shown in Tables I and II.

The normal Ca in rat's serum is 9.5 to 10 mg. per 100 cc., and the normal inorganic P is 7 to 8.5 mg. per 100 cc. In animals rendered rachitic by a diet low in phosphorus (such as diet No. 3143 of McCollum) the phosphorus is found to be about 3 mg. per 100 cc. and may run as low as 2 mg., while the calcium stays near 10 mg. per 100 cc. Fasting of such animals results in the healing of the rickets and a rise of the blood phosphorus to as much as 16 mg. per 100 cc.

* The writer wishes to express his sincere thanks to Dr. P. G. Shipley, Dr. E. V. McCollum, and Dr. Benjamin Kramer for their material help and advice during the course of these experiments.

TABLE I.
To Show the Effect of Fasting on the Ca and Inorganic P of the Serum of Rachitic Rats.*

Rats.		Fasted.	Returned to diet.	Ca	P	Product Ca × P	Weight before fasting.	Weight after fasting.	Weight at end of experiment.	Autopsy findings.
No.	Designation.									
3 (control).	R 24	None.		10	2.7	27.0				Active rickets.
	R 25									
	R 26									
1	R 4	2	0		16		108	92.5	92.5	Healing rickets.
2	R 7	2	0	5.4	16	86.4	151	132.5	132.5	"
	R 8						153	132	132	
2	R 19	4	0	6.2	11.5	71.3	164.5	137.5	137.5	Healing rickets.
	R 20						139.5	116.0	116.0	
2	R 2	2	2		2		106	89.5		Relapsing rickets. Calcified cartilage, healing rickets.
	R 3						104	88		
4	R 9	2	2	12	2.7	32.4	114		109	Relapsing rickets.
	R 10						120		119.5	"
	R 11						164		161.5	"
	R 12						105		100	"

The cause of this increase is uncertain. It might be supposed that destruction of tissue, for example muscle, might release a sufficient quantity of phosphorus to produce it. It is doubtful, however, if tissue destruction determines whether or not healing shall take place. Recent work in this laboratory, as yet unpublished, shows that animals may lose much weight without healing occurring. The striking fact is that without great loss in weight there is a return to the normal level, and in many instances an increase above it.

The experiments reported in this paper were designed to compare the reaction of normal animals to fasting with the reaction of rachitic animals to the same stimulus.

With this end in view the concentrations of Ca and inorganic P of the serum of rachitic and normal animals were determined at the end of varying periods of fasting. The same determinations were done with rachitic rats which, after a period of fasting, had received again their original diet for varying intervals of time.

Tables I and II are self-explanatory.

The bodies of these rats were examined after death and the condition of their bones was determined by microscopical examination.

It will be seen from Tables I and II that fasting induces a sharp rise in the level of the inorganic blood phosphorus. The calcium content of the blood, on the other hand, falls under the influence of the same treatment. The product of their concentrations, however, lies well above 40 (70 to 80, in fact). The lowest level of Ca which was determined by analysis was 5.4 mg. per 100 cc. In one instance (Rat 29) an animal died in convulsions, after 4 days fasting. Rachitic animals do not stand fasting as well as do normal rats in proportion to their apparent strength and activity at the beginning of the experimental period and we believe that the depression of the blood calcium is an important factor in their low resistance and perhaps in many cases the immediate cause of death.

It will be noted that animals returned to a rickets-producing diet after a period of fasting revert rapidly to the low blood phosphorus of the rachitic condition. The blood calcium undergoes a rapid augmentation, in some cases to above the normal level. No such wide variation of calcium and phosphorus in the serum of normal animals occurs during fasting. Since the serum of animals

fed on the stock diet contains 9.5 to 10.5 mg. of Ca and 7 to 8.5 mg. of P per 100 cc. it will be seen that the changes induced under the above experimental conditions were slight.

The mechanism by means of which these changes in the chemical composition of the blood in rachitic animals is brought about and the reason for the failure of normal animals to behave in the same way are unknown. Further attempts to study these phenomena are now in progress.

TABLE II.

To Show the Effect of Fasting on the Ca and Inorganic P of the Serum of Normal Rats.*

Rats.		Fasted.	Ca	P	Product Ca × P.	Weight before fasting.	Weight after fasting.	Remarks.
No.	Designation.							
		days	mg. per 100 cc.	mg. per 100 cc.		gm.	gm.	
2	Rn 3	On "stock diet."	9.9	7.8	77.2		253	Controls in metabolism cages 2 days. Normal bones.
	Rn 4						218	
2	Rn 1					252	237.5	Normal bones.
	Rn 2	2	8.8	7.5	66.0	219	199	
3	Rn 6					211	184.5	Normal bones.
	Rn 7	5	10.7	6.6	70.6	181	144	
	Rn 8					185	144	

* The animals described as normal in this paper were fed on McCollum's stock ration, which has the following composition:

	gm.
Wheat.....	25.0
Maize.....	25.0
Rolled oats.....	29.0
Flaxseed oil meal.....	10.0
Unpurified casein.....	10.0
CaCO ₃	0.5

Milk is given with this formula *ad libitum* and fresh cabbage twice per week under ordinary circumstances. These, however, were of necessity omitted from the diet during the short period in which the animals were in the metabolism cages. The diet with the milk omitted is somewhat low in calcium.

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CYSTINE DEFICIENCY AND VITAMIN CONTENT OF THE LENTIL, *LENS ESCULENTA* MOENCH.*

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WITH THE ASSISTANCE OF OTTO MOELLER.

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(Received for publication, January 7, 1924.)

The significance of the content of the various amino acids in the proteins in foods and feedingstuffs has become so generally recognized that the proportions of certain amino acids present are used as criteria by which the food value of proteins is judged. The ease with which the hexone bases, arginine, lysine, histidine, and cystine, can be determined by the Van Slyke method, as compared with the direct isolation method, has led to the frequent determination of these amino acids in various proteins. The relative importance of cystine in this group of amino acids has probably not been sufficiently emphasized. This amino acid differs characteristically from the other amino acids which have been shown to be essential for the normal growth and development of animals in that it contains sulfur.¹ Furthermore, the part which cystine plays in the oxidation and reduction processes in the living cell, as recently shown by Hopkins (1), gives this amino acid a unique place among the hydrolytic cleavage products of proteins. It has also been shown recently (2) that cystine or cysteine is essential in the culture medium used for the growth of certain bacteria.

* A preliminary report of this paper was presented at the meeting of the American Chemical Society held in Milwaukee, Wisconsin, September 10 to 14, 1923.

¹ Mueller has reported (Mueller, J. H., *J. Biol. Chem.*, 1923, lvi, 157) the isolation from casein of a new amino acid containing sulfur. As yet, however, we have no knowledge of its biological properties.

We have previously shown that the proteins of several legume seeds are nutritionally inadequate, chiefly because of deficiency in cystine. Albino rats made very unsatisfactory growth when fed diets in which navy beans (3), lima beans (4), adzuki beans (5), or cow-peas (6) constituted the sole source of protein. These diets were adequate with respect to the essential non-protein dietary factors. In most cases prompt decline in weight and death in a relatively short time followed. Aside from the cystine deficiency, these proteins are, furthermore, characterized by a form of indigestibility which can be corrected by cooking.

In this paper it is shown that the proteins of the lentil are likewise deficient in cystine and somewhat more efficient when cooked than when raw. The effect of cooking, however, is much less marked in the case of the lentil than in that of the proteins of the legume seeds mentioned, showing that the proteins of the lentil are the more digestible. This is in accord with results which other investigators have reported on the digestibility of lentil proteins. Rammstedt (7) found that raw lentil protein is more digestible than that of raw beans and has about the same digestibility as that of cooked pea meal. Berczeller (8) has reported that young rats lived for a comparatively short time on beans, much longer on peas, and longest on lentils. Studies on the specific value of certain feedingstuffs conducted by Völtz, Paechtner, and Baudrexel (9) gave results indicating that lentils have a relatively high digestibility coefficient.

Compared with the work done on other seeds of equal food importance, not much has been reported on the biological value of the proteins of the lentil. Osborne and Campbell (10) found the proteins of the lentil to be similar chemically to those of the pea, vetch, and horse bean.

Dry lentils, according to Chick and Delf (11), contain but little antiscorbutic vitamin, but when germinated they are a valuable source of this vitamin. This has been confirmed by Greig (12).

Cooper (13) found that lentils are a good and practical source of vitamin B.

The presence of vitamin A in lentils has not been heretofore shown, so far as we are aware, although in their paper on the antiscorbutic vitamin Chick and Delf state that the fat-soluble factor is probably deficient.

In connection with our work on the growth-promoting value of the proteins of lentils, the vitamin A and vitamin B contents were also determined. The results show that 2 gm. of lentils are about the minimum quantity needed daily to meet the vitamin B requirement of young albino rats, and that the quantity necessary to supply sufficient vitamin A lies not far from 2.5 gm. daily. The lentil appears, therefore, to be a much better source of this vitamin than most other seeds which have been examined.

The lentil, reported as being probably one of the first plants brought under cultivation by man, has been found in the ancient lake dwellings of St. Peter's Island, Lake of Biennne, which are of the Bronze Age (14). This seed is extensively used for food in all countries where it is grown, and is popularly regarded as being very nutritious. Although grown to a smaller extent in the United States than in many other countries, considerable quantities are imported annually.

The lentils used for the experiments described in this paper were obtained in the open market and were identified as the Large, Yellow Lentil, *Lens esculenta* Moench.² The seeds were ground to a fine meal and incorporated in the diets used. This meal contained 25 per cent of protein ($N \times 6.25$).

Care was taken to have all the rats used of nearly uniform weight and during the preexperimental period, subjected as nearly as possible to the same dietary and environmental conditions. No rats from litters of less than six were taken, and larger litters were reduced to eight as soon as possible after birth. Osborne and Mendel's (15) salt mixture was used in the diets and the cystine was prepared from wool. The rats were weighed twice weekly and their food intake was recorded.

Experiments with Raw and Cooked Lentil.

The striking inadequacy of the proteins of the raw lentil is shown on Chart 1, which gives the composition of the diet. Ample vitamin B was furnished by the 66 parts of lentils, and the 0.3 gm. of cod liver oil, given daily apart from the rest of the diet,

² We are indebted to Dr. D. N. Shoemaker of the Bureau of Plant Industry, United States Department of Agriculture, for the identification of these seeds.

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supplied vitamin A. The lentil furnished 16.5 per cent of protein. On this diet all the animals promptly declined in weight. Five of the six rats lived only from 17 to 49 days, an average of 33 days.

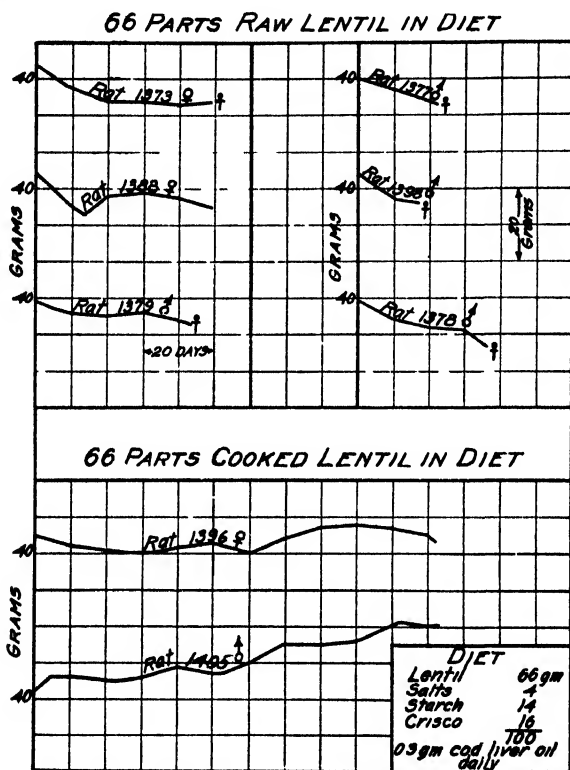


CHART 1.

The effect upon its nutritive properties of cooking the lentil is represented in the lower half of Chart 1. The diet used was the same as the raw lentil diet, except that the lentil meal had been first heated in an autoclave for 2 hours at 15 pounds pressure. After cooking, the meal was dried, ground, and incorporated in the diet. Rat 1396, a female, after 111 days had practically maintained its weight, and at the end of the same period rat 1405, a male, had gained 19 gm. It is apparent that cooking somewhat improved the nutritive properties of the lentil.

It has been previously found that the efficiency of the proteins of the navy bean, lima bean, velvet bean, and cow-pea in promoting growth is markedly increased by cooking. Digestion experiments *in vitro* showed that the raw proteins of the navy bean (3) and velvet bean (16) were difficultly digestible, and that cooking increased their digestibility. Although the digestibility of the proteins of the lentil has not been studied *in vitro*, there remains little, if any, doubt that the somewhat

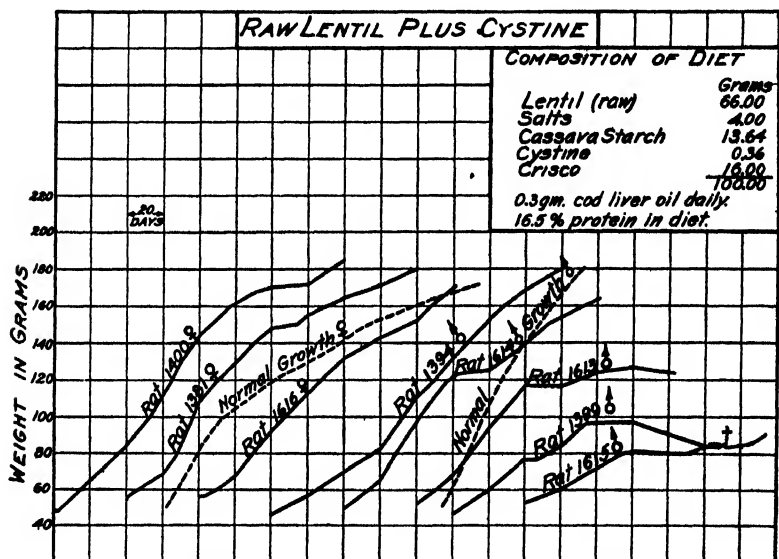


CHART 2.

better results obtained with the cooked than with the raw lentils were due to a higher degree of digestibility brought about by cooking. However, with the indigestibility factor removed, there remained another defect in the quality of the lentil proteins which permitted little more growth than mere maintenance.

Experiments with Raw and Cooked Lentil Plus Cystine.

The chief nature of the deficiency of the lentil proteins is shown by the results presented in Chart 2. The diet fed the rats whose growth curves are here represented differed from that shown in

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Chart 1 practically only in that 0.36 per cent of cystine was here incorporated. All the females grew at a rate better than normal. For some unknown reason the females grew much better than the males. The pronounced effect of adding 0.36 per cent of cystine to the raw lentil diet is apparent on comparing these curves with those of Chart 1. On the raw lentil diet to which no cystine had been added all the animals declined abruptly in

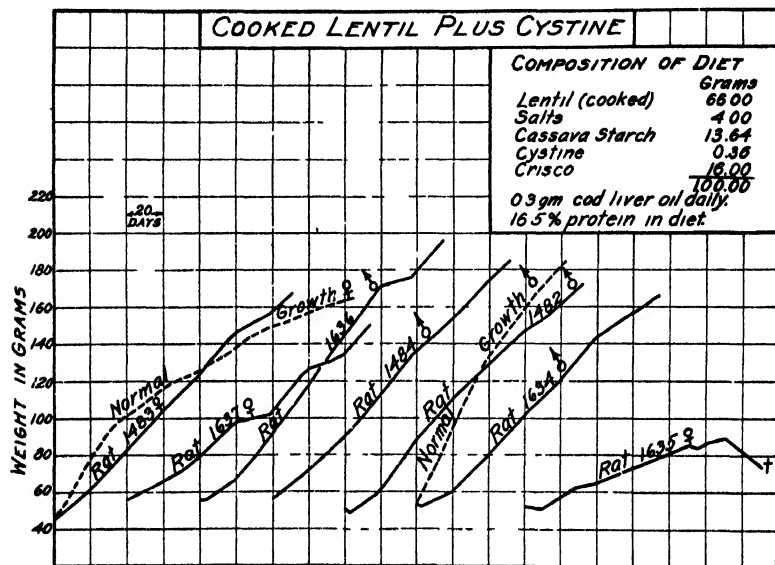


CHART 3

weight and lived but a relatively short time. On the same diet with cystine the majority of them made from fair to very good growths.

In Chart 3 are represented the rates of growth of rats fed the cooked lentil diet, supplemented with 0.36 per cent of cystine. As shown in Chart 1, somewhat better results were obtained with the cooked lentil meal than with the raw meal. Comparison of Charts 2 and 3 shows, however, that the animals made no better growths on cooked lentil plus cystine than on raw lentil plus cystine. But there was better utilization of the protein as measured by the gain in weight per gram of protein intake. The animals fed the raw lentil plus cystine diet had gained in

weight at the end of 110 days, an average of 0.66 gm. per gram of protein consumed, while on the cooked lentil plus cystine diet an average gain of 0.75 gm. was made.

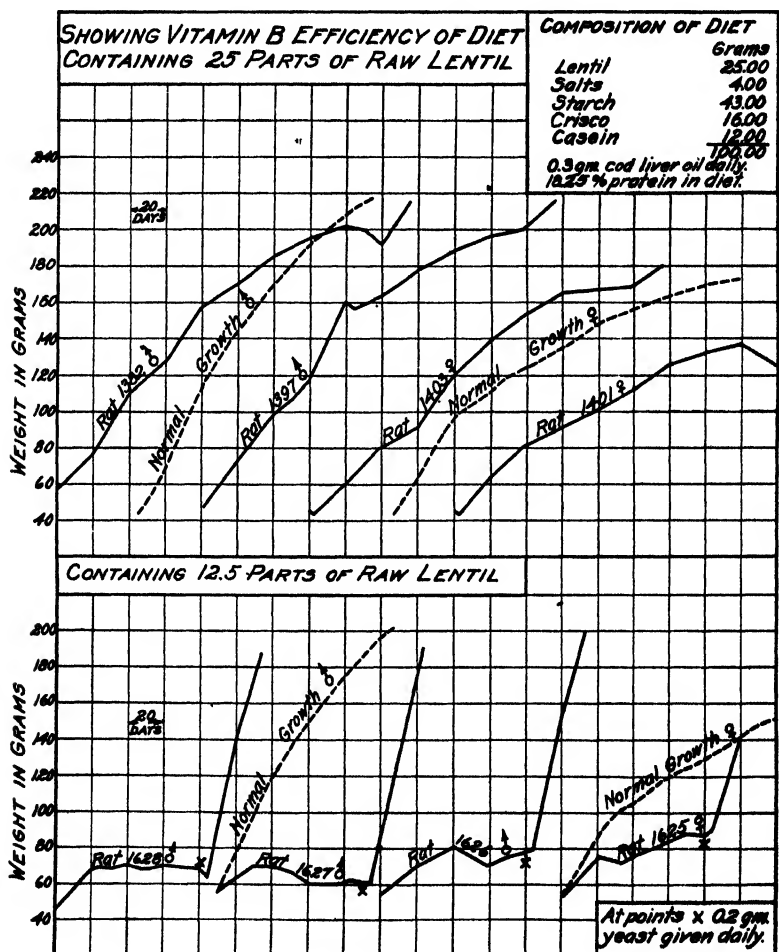


CHART 4.

Vitamin B.

The diets used in the determination of vitamin B contained 25 per cent of raw lentil meal as the sole source of this vitamin.

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To supply sufficient protein of good quality, casein³ was added. Control experiments had shown that the casein contained but little, if any, vitamin B. Each day 0.3 gm. of cod liver oil was given. When the lentil constituted 25 per cent of the diet, fairly good growths resulted (Chart 4).

Reducing the proportion of lentil to 12.5 per cent was followed by practically no growth after a slight initial gain during the first 20 days. After having been on the diet for 83 days, the emaciated animals were given 0.2 gm. of yeast daily. The response was striking. During the first 21 days, the period of initial growth, the average daily food intake of the rats receiving the diet containing 12.5 per cent was 8 gm. During this period, therefore, they received an average of 1 gm. of lentil daily. Since their food intake during this period was greater than it was after they had begun to decline in weight, it is evident that the quantity of vitamin B supplied by a daily allowance of 1 gm. of lentil enabled the rats to make only a very unsatisfactory growth.

The rats fed the 25 per cent lentil diet consumed daily during the first 100 days an average of 7.07 gm., or 1.77 gm. of lentil. On this quantity fairly good growths resulted. Although any numerical expression of the vitamin content of substance must as yet be considered approximate, we believe, after a careful analysis of the results of our experiments, that 2 gm. daily of lentil furnish about the minimum quantity of vitamin B required for the normal growth of the albino rat.

Vitamin A.

Preliminary experiments showed that 50 per cent of lentil in the diet furnished sufficient vitamin A. To get more definite data regarding the vitamin content of the lentil, two lots of rats were fed diets containing 25 and 12.5 per cent of the lentil.

³The casein used in these experiments was precipitated from fresh skim milk, washed with water, redissolved in dilute sodium hydroxide, and the solution filtered by suction through a thick mat of paper pulp. The casein was then reprecipitated with acetic acid. The treatment with sodium hydroxide and acetic acid was repeated twice. The product was then extracted successively in a copper extractor with alcohol and ether. The casein was finally heated in an electric oven at 110° for 24 hours.

The starch used was tested for vitamin A, but none could be detected.

Lot A (Chart 5), receiving 25 parts of ground lentil incorporated in the diet, grew at a rate well above normal for 80 to 125 days, with the exception of Rat 1690, a male, which made only a fair growth in 120 days. Ultimately, however, they declined in weight and all except Rat 1691, a male, which died suddenly, developed

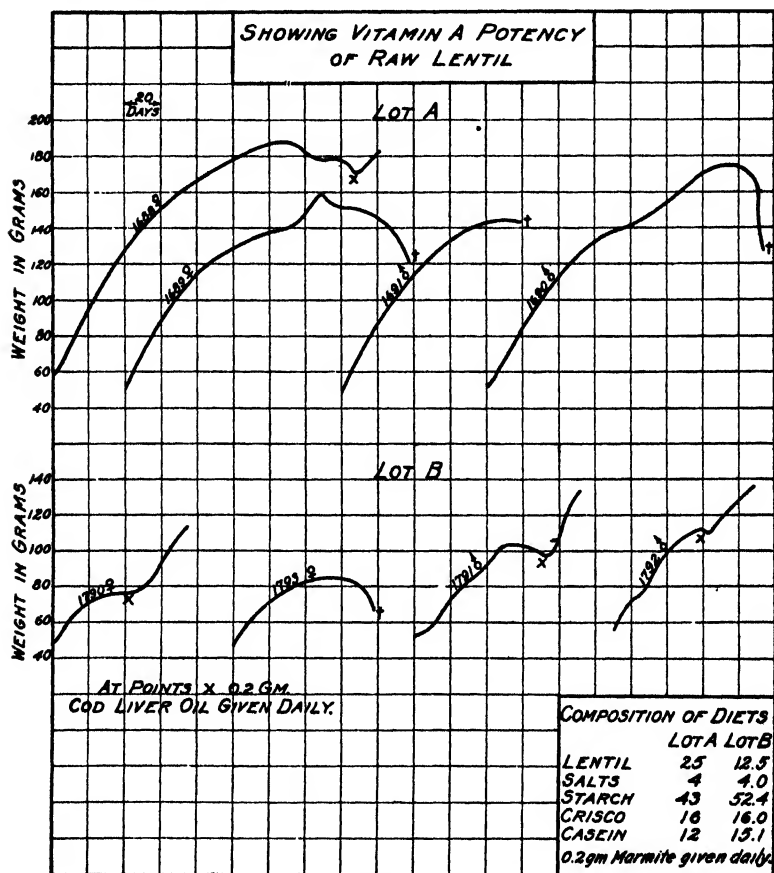


CHART 5.

sore eyes within 132 to 145 days. Rat 1688, a female, on the 167th day was given 0.2 gm. of cod liver oil. Prompt response in growth and complete recovery from xerophthalmia resulted.

When the lentil content of the diet was reduced to 12.5 per cent (Lot B), the animals made only an initial growth for 29 to

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49 days, and in every case developed xerophthalmia, the symptoms appearing in 40 to 60 days. When positive symptoms of the eye trouble were manifested, and in some cases had advanced to a severe degree, 0.2 gm. of cod liver oil was given daily. With the exception of Rat 1793, a female, which had gone too far to recover, all responded promptly. The eyes were completely cured and growth at a good rate was resumed.

The satisfactory rate at which the rats grew for comparatively long periods when fed 25 parts of lentil in the diet indicates that they were receiving a quantity of vitamin A not far below their requirements.

The importance of keeping a record of the food intake of each rat in all work for evaluating the vitamin content of any material is well brought out in these experiments. The average daily food intake of the animals receiving the 25 per cent lentil diet (Lot A), calculated from the beginning of the experiment to the time when growth ceased, was 9.2 gm. They consumed, therefore, an average of 2.3 gm. of lentil daily. The daily food intake of those on the 12.5 per cent lentil diet (Lot B), similarly calculated, was 5.25 gm., equivalent to 0.65 gm. of lentil. The rats in Lot B, although receiving a diet containing one-half as much lentil as those in Lot A, were actually getting of the lentil only about one-fourth as much. To express, therefore, as is sometimes done, the vitamin value of any substance in terms of the percentage in which that substance is found in the diet, has little, if any, significance unless the food intake is also taken into consideration.

SUMMARY.

The proteins of the lentil, *Lens esculenta*, like those of the beans of the genus *Phaseolus*, are limited in their nutritive value by a deficiency of cystine. Albino rats fed a diet containing 66 per cent of raw lentil declined in weight promptly and lived, on an average, only 33 days. Somewhat better results were obtained when the lentil had been cooked. On the same diet to which 0.36 per cent of cystine had been added the animals made from fair to very good growths.

2 gm. daily of lentil furnish about the minimum quantity of vitamin B required for the normal growth of the albino rat, while the quantity needed to furnish the required vitamin A is not far from 2.5 gm.

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SOME APPLICATIONS OF THE COLORIMETRIC PHOSPHATE METHOD.

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Two years ago, a modification (1) of the Bell-Doisy phosphate method was published and at that time technique was given for its application to inorganic phosphorus in blood and urine. Since then its use has been extended to a variety of analytical procedures, some of which are given below.

In principle the method consists of the formation of phosphomolybdic acid and its subsequent reduction by hydroquinone and sulfur dioxide with the production of a stable blue color, proportional to the inorganic phosphorus present. The excess molybdic acid is not reduced.

Reagents.—The following reagents are used in all of the procedures to be given.

Molybdate Solution.—This contains 5 per cent ammonium molybdate in 5 N H_2SO_4 , and is prepared as follows: Dissolve 25 gm. of ammonium molybdate in 300 cc. of water and to this add 75 cc. of concentrated H_2SO_4 diluted with 125 cc. of water.

Hydroquinone Solution.—This is a 1 per cent solution to which a drop of concentrated H_2SO_4 is added to retard oxidation.

Sulfite Solution.—A 20 per cent solution of sodium sulfite is used. The strength of sulfite solutions deteriorates by oxidation, but the amount used in color development is sufficient provided an easily detectable odor of SO_2 is evolved.

Stock KH_2PO_4 Solution.—This is prepared from pure KH_2PO_4 which has been pulverized and dried for several days over concentrated H_2SO_4 . 4.394 gm. are dissolved in a liter of distilled water and then 5 cc. of CHCl_3 added as preservative. 1 cc. = 1 mg. of phosphorus.

Standard KH_2PO_4 Solutions.—The following dilutions of the stock solution are prepared for standards in the various procedures. 100 cc. of the stock solution to a liter of water. 1 cc. = 0.1 mg. of phosphorus. From this solution three additional dilutions are made.

50 cc. to 200 cc.	1 cc. = 0.025 mg. P.
50 " " 250 "	1 " = 0.02 " "
50 " " 500 "	1 " = 0.01 " "

Each of these solutions is preserved by $CHCl_3$.

Phosphorus Compounds of the Blood.

I. Inorganic Phosphorus in Blood or Plasma.

The method previously published seems to have proved quite satisfactory in every respect except the intensity of the color produced. By a few slight changes the color has been considerably improved and the proportionality to inorganic phosphorus unimpaired. A much better color is obtained if the order of adding the sulfite and hydroquinone is reversed, that is by adding the molybdate and hydroquinone and then the sulfite after the green color appears. The development of color is retarded by large amounts of acid as was pointed out before. By using 1 cc. of the acid molybdate solution instead of 2 cc. an abundant excess of molybdate is provided and the diminished acidity allows the reduction to proceed to a much greater extent in the half hour allowed. The procedure, then, which seems best at present, is: Transfer a measured volume of blood or plasma to a small flask. Add 3 volumes of water and 1 volume of 20 per cent trichloroacetic acid, shake vigorously for a minute, and pour onto an ashless filter. Transfer 5 cc. of the filtrate to a 10 cc. graduated cylinder, to another cylinder transfer 3 cc. = 0.03 mg. of P of a standard KH_2PO_4 solution. To each then add in the following order, 1 cc. of molybdate, 1 cc. of hydroquinone, and 1 cc. of sulfite. Dilute to 10 cc. and after a half hour compare. Set the standard at 30 mm.

By using a molybdate solution prepared with less H_2SO_4 , colors of still greater intensity may be obtained. If this is done trichloroacetic acid equivalent to that in the filtrate should be

added to the standard, so that reduction will proceed at the same rate.

In spite of the exposure to strong mineral acid, the results obtained by this method are not higher than those reported by others (2, 3). Apparently there is very little hydrolysis of organic phosphate. In order to get more definite information in regard to this question, comparison was made with the method of Bloor (2) which has been used by Buell (4) and others where it was desired to avoid hydrolysis of organic phosphates. A mixture was made from ten samples of blood brought into the routine laboratory. Duplicate determinations by the colorimetric technique above gave identical results, 4.50 mg. of P per 100 cc. Duplicate determinations by Bloor's method gave 4.46 and 4.44 mg. per 100 cc. These results confirm the notion that there is very little hydrolysis by the colorimetric reagents.

II. Inorganic Plus Hydrolysable Organic Phosphorus.

If the filtrate and inorganic reagents are heated in boiling water the results are a little higher for plasma and a great deal higher for whole blood, due to the presence of a labile organic phosphate reported by Zucker and Gutman (5). Inorganic plus hydrolysable organic phosphorus may be determined conveniently by the following technique. Transfer 5 cc. of the trichloroacetic filtrate from plasma (or 2 cc. of whole blood filtrate plus 3 cc. of water), to a test-tube graduated at 10 cc. To a similar tube transfer 3 cc. of standard KH_2PO_4 solution equivalent to 0.03 mg. of P and add to this tube 2 cc. of water. Then to each tube add 1 cc. of molybdate, 1 cc. of hydroquinone, and 1 cc. of 10 N H_2SO_4 ; mix these solutions well with a stirring rod, as the acid molybdate tends to layer in the bottom. Rinse off the stirring rod, insert rubber stoppers loosely into the tubes, and immerse them in boiling water for 15 minutes (an hour for whole blood filtrates). Cool to room temperature under the tap and then add to each tube 1 cc. of the sulfite solution and dilute to 10 cc. Mix and after 10 minutes compare in the colorimeter.

The 10 N H_2SO_4 may be prepared with sufficient accuracy by diluting 30 cc. of concentrated H_2SO_4 with 70 cc. of water. If too much acid is added the reduction by hydroquinone is pre-

vented and if too little acid is present molybdic acid as well as phosphomolybdic acid will be reduced. Sulfite should not be added until after the heating, as different amounts of SO_2 are driven off from the separate tubes with the result that different degrees of reduction are obtained and the colors produced do not match.

Since the amount of hydrolysable phosphate in plasma is small, it has been recommended by Myers (6) that a similar technique be used for the clinical determination of inorganic phosphorus in serum. Analysis of three human plasmas for inorganic phosphorus and inorganic plus hydrolysable phosphorus gave results shown in Table I.

TABLE I.

No.	Phosphorus per 100 cc. plasma.	
	Inorganic P.	Inorganic + hydrolysable P.
	mg.	mg.
I	2.88	3.05
II	2.91	3.20
III	3.32	3.48

These results indicate that values about 10 per cent higher may be expected where reduction is accelerated by heat.

III. Total Acid-Soluble Phosphorus.

This may be determined colorimetrically on a small amount of the trichloroacetic filtrate digested with nitric and sulfuric acids. For accurate results it is necessary to drive off all the nitric acid and very little of the sulfuric acid. By substituting 30 per cent H_2O_2 for nitric acid the digestion is very much facilitated; Merck's White Label Superoxal has been used.

Procedure.—Transfer 2 cc. of the trichloroacetic filtrate to a large Pyrex tube graduated at 25 cc. and add 1 cc. of 10 N H_2SO_4 . Drop in a glass bead and heat over a micro burner until the fumes of SO_3 appear. Turn off the flame and allow the tube to cool for about a minute. Add a drop of 30 per cent H_2O_2 , cover the tube with a watch-glass and heat over the micro burner for 10 minutes, adjusting the flame rather low so as to avoid loss of SO_4 . Cool the tube a few seconds in running water and dilute with 18 cc. of distilled water. To a similar tube transfer 10 cc.

of a standard KH_2PO_4 solution = 0.1 mg. of P, 1 cc. of 10 N H_2SO_4 , and 7 cc. of distilled water. To each tube add 2 cc. of the molybdate solution and 2 cc. of the hydroquinone solution. Cover the tubes with small beakers and heat a half hour in boiling water. Cool, add 1 cc. of the sulfite, dilute with water to 25 cc., mix, and after 10 minutes compare.

The oxidation of charred carbonaceous material is very prompt after the addition of a drop of the 30 per cent H_2O_2 . The heating for 10 minutes is directed to decompose excess peroxide, which if present would interfere with color production.

Lipoid Phosphorus.

Randles and Knudson (7) have given a procedure for the determination of lipoid phosphorus using the original Bell-Doisy method of color development. By application of the technique given above for total acid-soluble phosphorus to the evaporated residue from an aliquot of the alcohol-ether filtrate one gains the advantage of the stable acid color for comparison.

Determination of Calcium as Phosphate.

Calcium may be determined on 5 cc. of serum by the oxalate-permanganate method with an error of ± 5 per cent. When the analysis is carried out on smaller amounts of material the error is correspondingly greater, due chiefly to limitations of the permanganate titration.

Upon considering the possibility of determining small amounts of calcium as phosphate the following points were observed.

1. Very small quantities of calcium may be completely precipitated with a moderate excess of oxalate.

2. The precipitate could be separated by centrifugation and decomposed with a drop of concentrated HCl and a few drops of 30 per cent H_2O_2 .

3. The calcium could be precipitated from the resulting small volume of solution by the addition of a few drops of a phosphate solution and a few drops of strong ammonia.

4. The calcium phosphate could be washed with ammoniacal 20 per cent alcohol and the phosphate determined colorimetrically.

5. Calcium forms a variety of phosphates, their composition depending upon the alkalinity and the relative amounts of CaO and H_3PO_4 present (8). However, when a dilute acid solution of calcium containing excess phosphate is made alkaline with ammonia, the precipitate formed contains an amount of phosphorus corresponding very closely to the formula $\text{Ca}_3(\text{PO}_4)_2$.

These principles are utilized in the following procedure, an application to blood or plasma. Transfer 10 cc. of the trichloroacetic filtrate (= 2 cc. of plasma) to a Pyrex test-tube (16 mm. \times 150 mm.), graduated at 15 cc. Add a drop of methyl red as indicator and while agitating with a rubber tipped stirring rod add dilute ammonia (concentrated ammonia diluted with 3 volumes of water) until the color changes to yellow, then add a few drops of 5 per cent acetic acid to produce the neutral brown. Add 1 cc. of 4 per cent ammonium oxalate and rub the sides of the tube with rubber tipped rod until the precipitate forms. Rinse off the stirring rod and allow 2 hours standing for complete precipitation. Centrifugate 10 minutes at 1,500 R.P.M. and pour off the fluids into a similar tube for the determination of magnesium. To the residue of calcium oxalate add 1 drop of concentrated HCl and 0.5 cc. of 30 per cent H_2O_2 and heat 30 minutes in boiling water. Then add 0.5 cc. of 2 per cent KH_2PO_4 and 3 drops of concentrated ammonia (or 1 drop after the precipitate forms). Allow 30 minutes standing for complete precipitation, then add 20 cc. of the wash solution, centrifugate 10 minutes at 1,500 R.P.M., and pour off the fluids. Add 20 cc. of the wash solution, then with the rubber policeman scrub the sides of the tube and mix up the precipitate. Centrifugate again and pour off the fluids. To the residue of $\text{Ca}_3(\text{PO}_4)_2$ add 5 cc. of water and to a similar tube add 5 cc. of a standard KH_2PO_4 solution equivalent to 0.1 mg. of P. To each add 1 cc. of the molybdate, 1 cc. of the hydroquinone, and 1 cc. of the sulfite. Dilute with water to 15 cc. and after a half hour compare. 1 mg. of P is equivalent to 1.935 mg. of Ca.

Reagents.—30 per cent H_2O_2 , Merck's White Label Superoxal was found satisfactory. The wash solution contains 200 cc. of 95 per cent alcohol and 50 cc. of concentrated ammonia per liter.

Analysis of known calcium solutions by this procedure gave theoretical results. In Table II are given comparative results by the permanganate and colorimetric methods.

Remarks.—Where precipitates are to be separated by centrifugation and “pouring off” of fluids, it is desirable to have the insoluble material in a very finely divided state in order that the forces of cohesion may be more effective. When calcium oxalate or magnesium ammonium phosphate are “rubbed down” a very finely divided precipitate is formed, in contrast to the relatively coarse grained precipitates obtained when they are allowed to form on standing. Calcium phosphate is precipitated at once on adding excess ammonia, but in a finely divided form, well suited to the technique of separation described. Calcium phosphate is of course not so insoluble in water as calcium oxalate, but this is offset by the use of alcohol as a wash fluid. After pouring off twice as described there remains less than 0.001 mg. of soluble phosphate.

TABLE II.

Analysis of Trichloroacetic Beef Serum Filtrate.

By permanganate	By colorimetric.
10.3 mg. per 100 cc.	10.20 mg. per 100 cc.
10.3 “ “ 100 “	10.30 “ “ 100 “
10.0 “ “ 100 “	9.92 “ “ 100 “
	10.13 “ “ 100 “
	9.92 “ “ 100 “
	9.80 “ “ 100 “
	9.92 “ “ 100 “
	9.99 “ “ 100 “

Determinations by the permanganate method were made on 25 cc. of the filtrate, equivalent to 5 cc. of serum. For the determinations by the colorimetric method 10 cc. portions were used.

Determination of Magnesium.

The technique in use at present for blood is as follows: To the fluid poured off from the calcium oxalate precipitate, and received in a Pyrex test-tube (16 × 150 mm.), graduated at 15 cc. add 1 cc. of 2 per cent KH_2PO_4 and 1 cc. of concentrated ammonia. Rub down the precipitate with a rubber policeman as described for calcium oxalate, rinse off the policeman, and allow 4 hours standing for complete precipitation. Centrifugate 10 minutes at 1,500 R.P.M. and pour off the fluids. Wash with 20 cc. of the alcoholic wash fluid as described for calcium phosphate.

Centrifugate again and pour off. Dissolve the precipitate and develop the color as described for calcium. For a standard use 3 cc. of a KH_2PO_4 solution equivalent to 0.075 mg. of P or 0.0588 mg. of Mg.

Magnesium in Urine.

A rapid and accurate technique for determining magnesium without separation of calcium is as follows: Transfer 1 or 2 cc., depending on the amount of magnesium expected, of clear acid urine to a Pyrex test-tube similar to that described above. Add a drop of methyl red and then dilute ammonia drop by drop until the color changes to brown. Adjust with a few drops of 5 per cent acetic acid if too much ammonia is added. Add 1 cc. of 4 per cent ammonium oxalate and rub down the calcium oxalate; after 2 hours standing add 1 cc. of 2 per cent KH_2PO_4 , 1 cc. of concentrated ammonia, and rub down the MgNH_4PO_4 . Allow 2 hours standing for complete precipitation. Wash twice with the alcohol wash solution by centrifugation and pouring off as described for calcium on blood. Dissolve the combined calcium and magnesium precipitates in a little hydrochloric acid approximately 0.2 N. Develop the color as described for magnesium on blood. Compare with a standard containing 0.10 mg. of P = 0.0784 mg. of magnesium.

Results reported before (9) by a similar technique in which CaC_2O_4 and MgNH_4PO_4 were precipitated at the same time were a little high, due no doubt to the formation of some calcium phosphate. Results by the technique described here give results which check gravimetric determinations within ± 2 per cent.

Determination of Total Base.

Total base has been determined recently by Van Slyke and coworkers (10) as sulfate. All other acids except phosphoric acid were expelled by heating with excess sulfuric acid; the phosphoric acid present was converted to *m*-phosphoric acid, a monovalent acid. The base bound by phosphoric acid was determined separately and added to that computed from the residue of sulfates.

A convenient procedure suggested by that above is to add an excess of phosphoric acid to the combined sulfates. Then by

heating at high temperature drive off all the sulfuric acid and some of the excess phosphoric acid. The excess of phosphoric acid is titrated and the total phosphates are determined colorimetrically. After reducing the results to equivalent normal solutions the total base is obtained by difference.

The actual technique as applied to a urine is: Transfer 5 cc. of a clear acid urine to a platinum crucible and evaporate cautiously to dryness. Moisten with a drop or so of concentrated H_2SO_4 and ignite gently. Cool, moisten with sulfuric acid, and again ignite. Repeat if the ash is not white, but avoid heating to redness at this stage. Add 0.2 cc. of a phosphoric acid solution equivalent to approximately 50 mg. of phosphorus. Heat over a free flame, cautiously at first, until the SO_3 has been driven off and the phosphoric converted to *m*-phosphoric acid. Then heat so that the whole crucible is kept at a dull red heat for 40 to 60 seconds. Cool, add 10 cc. of standardized 0.1 N H_2SO_4 . Cover with a watch-glass and heat 2 hours on a water bath, adding water if necessary. Transfer to a small flask and titrate with standard 0.1 N NaOH using methyl red as indicator and as a color for the end-point, a similar flask containing the same amount of water and methyl red, and about 10 mg. of pure KH_2PO_4 . Then transfer the titrated contents to a 100 cc. volumetric flask, dilute with water to the mark and mix. Transfer 10 cc. of this solution to a 200 cc. volumetric flask. To a similar flask transfer 5 cc. of the stock KH_2PO_4 solution equivalent to 5 mg. of P. To each add 25 cc. of the molybdate solution, 20 cc. of the hydroquinone solution, and dilute with water to 200 cc. After a half hour compare in the colorimeter.

A very intense green color is obtained for comparison so that the addition of sulfite is unnecessary.

The phosphoric acid solution used is that obtained by mixing 3 volumes of 85 per cent phosphoric acid and 1 volume of concentrated H_2SO_4 . After the precipitated calcium has settled, the phosphoric acid content was determined roughly to be about 50 mg. of P for 0.2 cc.

Calculation.—The normal equivalent of the 10 cc. of 0.1 N H_2SO_4 used to accelerate solution and hydrolysis is subtracted from the normal equivalent of the titration. This value is then subtracted from that obtained by dividing the total mg. of

phosphorus by 31.04. This answer is, of course, cubic centimeters of normal base per 5 cc. of urine.

Three determinations by this procedure on 30 mg. of Na + 5 mg. of Ca, equivalent to 15.5 cc. of 0.1 N base, gave 15.13, 15.62, and 15.60 cc. of 0.1 N base. Duplicate determinations on a urine by this procedure gave 124 and 130 cc. of 0.1 N base per 100 cc.; by the method of Van Slyke, 124 and 125 cc. of 0.1 N base.

SUMMARY.

Improved technique is given for the colorimetric determination of small amounts of inorganic phosphate. Procedures are given for the application of the colorimetric phosphate method to the determination of various phosphorus compounds of the blood, for calcium and magnesium, and for total base.

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STUDIES OF THE EFFECT OF EXERCISE IN DIABETES.

I. CHANGES IN ACID-BASE EQUILIBRIUM AND THEIR RELATION TO THE ACCUMULATION OF LACTIC ACID AND ACETONE.

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The researches of Fletcher and Hopkins (1) on surviving muscle and the later investigations of Embden (2), A. V. Hill (3), Meyerhof (4), and others have established the normal chemical mechanism of muscular contraction as a breakdown of glycogen or some intermediary carbohydrate into lactic acid. This occurs during the phase of shortening of muscle in the presence or in the absence of oxygen. When oxygen is present, a rapid disappearance of lactic acid follows the contraction. Evidence of the production and disappearance of lactic acid appears whenever a normal man does vigorous muscular exercise. Accompanying the exertion there is a rapid accumulation of lactic acid in the circulating blood and a marked change in the acid-base equilibrium which is directly attributable to the increase in lactic acid (5). The presence of large amounts of lactic acid in the circulation is only temporary. Usually, within an hour from the end of exercise all the excess lactic acid has disappeared. Its fate after exertion is not definitely known. Only a small portion is excreted (6). Barr and Himwich (7) found that following leg exercise, lactic acid rapidly disappeared during the passage of the blood through the non-exercising tissues of the arm so that each 100 cc. of the returning venous blood contained 10 to 25 mg. less of lactic acid than the arterial blood which supplied the arm. Whether this evidenced a distribution of lactic acid between blood and tissues or whether the

lactic acid was oxidized or converted into some other substance could not be shown. The work of Meyerhof (8) might indicate that it is chiefly reconverted into glycogen or some carbohydrate precursor.

The response of diabetic individuals to muscular exercise has not been studied from the standpoint of the accumulation and removal of lactic acid. Theoretically, such an investigation presents considerable interest in connection with the general question of the mechanism of muscular contraction in individuals whose ability to oxidize carbohydrate is limited or lost. Practically, it is of importance in regard to the effect of exertion upon the physical well-being of diabetics and to the advisability of allowing them to exercise.

Theoretic Considerations.

In all the experimental work in the physiology of muscle, the breakdown of carbohydrate into lactic acid is the only chemical mechanism of contraction for which there is convincing evidence.

In their recent article on muscular exercise Hill and Lupton¹ express the view of many physiologists: "It would seem probable that carbohydrate alone provides the energy for the excess metabolism of exercise." The question arises whether this can apply to the severe diabetic whose illness consists of his inability to metabolize carbohydrate. There is a considerable amount of evidence which indicates that under these circumstances some other mechanism must be employed.

In the first place, the ability of the diabetic organism to convert carbohydrate into lactic acid has been questioned. The perfusion experiments of Embden and Isaac (10) indicate that in some phlorhizinized and in all their depancreatized dogs, the liver loses its normal function of changing glucose into lactic acid. Lépine (11) found in depancreatized dogs and in a patient with severe diabetes that glycolysis was greatly diminished in the blood. More recently, Thalheimer and Perry (12) and Denis and Giles (13) have published observations on human subjects which seem to confirm this and to indicate that the diminution in glycolysis

¹ Hill and Lupton (9), p. 141.

parallels to some extent the severity of the diabetes. However, in a paper shortly to be published in this Journal, Tolstoi² finds no difference between the glycolytic power of normal and diabetic blood when kept at 38°C. Woodyatt (14) studied the lactic acid formation in muscles removed from normal and diabetic dogs and from a patient with "bronzed" diabetes. In the muscles of the phlorhizinized dogs, the lactic acid maximum was only one-third of the yield in normal dogs. In the human diabetic it was even less. von Fürth (15), however, in similar experiments, was unable to demonstrate any consistent difference between the lactic acid formation in normal and diabetic muscles from human subjects. The latter were obtained from patients dying in diabetic coma. Parnas (16, 17) found the same amount of lactic acid in the muscles of a normal and a diabetic frog. In muscle experiments, Embden and coworkers (18) showed that completely phlorhizinized dogs and rabbits had lactic acid in the freshly removed muscles and that more lactic acid was formed when these muscles were allowed to stand. Even after strychnine convulsions in a fasted and phlorhizinized dog, lactic acid could be demonstrated in considerable amounts.

In considering the foregoing results, it appears that with the exception of the liver perfusion experiments of Embden, the evidence indicates that in diabetes there may be a diminution but not a complete loss of the function of converting glucose into lactic acid. In all the observations of Lépine, Thalheimer and Perry, and Denis, there is some glycolysis in the blood. One of Woodyatt's phlorhizinized dogs in which there was a complete D:N ratio and in whose muscles no glycogen was found, still had a lactic acid formation about one-third of that recoverable from the muscles of a normal dog. von Fürth in one of his patients, dead from diabetic coma, obtained a lactic acid yield exceeding the highest of his normals.

It has been suggested as a possibility, notably by Bayliss (19), that muscular contraction is not dependent upon the formation of lactic acid as such, but is accomplished by the resultant accumulation of hydrogen ions which might occur as well through the agency of some other acid, such as β -hydroxybutyric or diacetic acid. It

² Personal communication.

seems possible that these acids might be formed in large amount during exercise in severe diabetics. The respiratory quotients of such individuals at rest indicate that fat is chiefly utilized. The experiments of Grafe and Salomon (20) demonstrate that the utilization of fat continues during exercise. In fact, their observations reveal a lower respiratory quotient during exercise than before. Unpublished experiments by Richardson and Ladd (21) confirm this. If the respiratory quotients can be accepted as actually representing the proportion of fat and of carbohydrate which is oxidized, there seems to be no escape from the conclusion that in the diabetic, muscular work is performed chiefly at the expense of fat. During the exertion, the metabolism is much higher than at rest. Judging from the quotients the extra heat production is accomplished by a marked increase in fat combustion without a corresponding rise in the oxidation of carbohydrate. According to prevailing ideas of ketogenic-antiketogenic balance, this would lead to the production of β -hydroxybutyric and diacetic acids which might, if the hypothesis suggested by Bayliss is correct, be utilized in the production of muscular contraction.

If lactic acid is formed the rate and mode of its disappearance in the diabetic are also of interest. The experiments of Mandel and Lusk (22) upon phlorhizinized dogs and dogs poisoned by phosphorus indicate that the normal body may readily accomplish the conversion, $\text{glucose} \rightleftharpoons \text{lactic acid}$ in both directions. The experiments of Embden and Isaac (10), already mentioned, indicate that the diabetic animal loses the power of converting glucose to lactic acid. On the other hand, they demonstrate that the opposite reaction—lactic acid to glucose—is retained. On theoretic grounds, therefore, one might expect that any lactic acid which was formed would be readily reconverted into a carbohydrate precursor and that it would disappear from the circulating blood at least as rapidly as in normal individuals.

The problem of acid formation in the diabetic during exercise is of practical as well as of theoretic importance. The temporary accumulation of lactic acid in normal, exercising individuals is not detrimental. If the effect of exercise can be taken as a criterion it seems actually to be beneficial. In the diabetic, however, with his constant tendency to acidosis, the acid which is added during exercise may diminish still further the depleted alkali

reserves of the body. This might possibly cause damage if the accumulating acid is lactic and most certainly would be detrimental if there is an increase of acetone bodies.

Interest in these questions led us to make certain observations on the response to exercise of several diabetics. In this, the first paper of a short series, an attempt will be made to answer the following questions: (1) Are the changes in acid-base equilibrium during exercise in diabetes similar to those which have been observed in normal individuals? (2) Is lactic acid accumulated in the circulating blood? (3) Can the changes in acid-base balance be accounted for quantitatively by lactic acid accumulation? (4) Is there an accumulation of acetone bodies in the blood? (5) If accumulated in the blood stream, does lactic acid disappear as in normal individuals? (6) Is exercise detrimental to the diabetic? To this last question the nature of our data allows us to give only a most incomplete answer.

Methods.

A complete description of the methods employed in the study of the acid-base equilibrium may be found in an article previously published (5). CO_2 and oxygen were determined by the method of Van Slyke and Stadie (23). In several of the experiments, in which there was a limited amount of blood, the Lundsgaard and Möller (24) modification of the Van Slyke-Stadie technique for oxygen capacity was employed. Since in our hands this method gave somewhat lower results than the earlier technique, note is made in the protocols at the end of the paper whenever the modification was used. pH was calculated as in our previous experiments by the Hasselbalch equation (25) using a pK_1 of 6.10. In spite of the increasing evidence that the pK_1 for whole blood is higher than 6.10, this value has been retained in order that the results in diabetics during exercise may be directly comparable to the previously published observations on normal subjects. Lactic acid was obtained by the method of Clausen (26). Precipitation of blood for this determination was performed immediately because of the tendency to glycolysis and consequent formation of lactic acid in blood which is allowed to stand outside of the body. According to Evans (27), glycolysis may be prevented by sodium

TABLE I.
Changes in Acid-Base Equilibrium Resulting from Short Periods of Exercise.

Subject.	Date.	Time in relation to exercise.	CO ₂ capacity at 40 mm. CO ₂ tension.	CO ₂ tension of arterial blood.	pH of arterial blood.	Remarks.
William R.	1923 Oct. 31	Before.	37.0	30.0	7.30	Exercise = 1,764 kg.m. in 3½ min.
		After.	27.5	35.5	7.10	
	Nov. 16	Before.	47.5	43.6	7.29	Exercise = 2,185 kg.m. in 3 min.
		After.	36.0	37.0	7.21	
Chris Q.	Nov. 21	Before.	45.0	46.0	7.26	Exercise = 2,240 kg.m. in 3½ min.
		After.	36.0	37.8	7.20	
	Dec. 1	Before.	45.5	46.0	7.26	Exercise = 2,355 kg.m. in 3½ min.
		After.	33.5	38.5	7.17	
Ben C.	Dec. 13	Before.	40.6	46.5	7.21	Exact exercise not recorded. Approximately 2,000 kg.m. in 3½ min.
		After.	31.4	34.8	7.16	
	Dec. 22	Before.	42.0	46.5	7.22	Exact exercise not recorded. Approximately 2,000 kg.m. in 3½ min.
		After.	33.5	40.6	7.15	
1923 Feb. 3		Before.	48.8	43.0	7.32	Exercise = 1,386 kg.m. in 3½ min.
		After.	36.3	40.0	7.20	

Jervis B.	Apr. 5	Before.	43.6	51.5	7.21	Exact exercise not recorded. Approximately 1,500 kg.m. in 3½ min.
		After.	35.7	45.5	7.17	
James F.	Apr. 10	Before.	44.5	48.0	7.24	Exact exercise not recorded. Approximately 1,500 kg.m. in 3½ min.
		After.	34.0	39.0	7.18	
George H.	Apr. 19	Before.	42.0	41.0	7.26	Exact exercise not recorded. Approximately 2,000 kg. m. in 3½ min.
		After.	32.5	38.0	7.15	
	Apr. 26	Before.	48.2	38.8	7.35	Exercise = 2,004 kg.m. in 3½ min.
		After.	37.2	34.8	7.24	

fluoride. As an added precaution against *in vitro* lactic acid formation, sodium fluoride in 0.1 per cent solution was utilized in amounts calculated to furnish 0.02 mg. of fluoride per cc. of blood. Correction for acetone in the lactic acid method was made in the manner advised by Clausen. All the figures in the protocols and tables represent the values for lactic acid after the correction for acetone has been made. Total acetone bodies were determined by the method of Van Slyke (28). Throughout this paper the acetone figures indicate that 1 mg. of precipitate has been taken as the equivalent of $\frac{1}{10}$ mg. of acetone. All exercise was performed on a Krogh bicycle ergometer. Samples of blood were taken from the brachial artery or an arm vein before exercise and usually from 2 to 4 minutes after the exertion.

EXPERIMENTAL.

Brief descriptions of the patients whom we have studied will be found at the end of the paper. Most of them were being studied simultaneously in the metabolism ward and calorimeter of the Russell Sage Institute of Pathology. Several were suffering from a severe form of the disease, but none of them was a complete diabetic. In two instances, the patients were receiving insulin on the day on which our observations were made (Jervis B., April 5, 1923 and Ben C., February 3, 1923).

Changes in Acid-base Equilibrium Following Exercise.

Eleven observations on six diabetics were made to determine the changes in CO_2 -combining capacity, CO_2 tension, and pH which occurred in arterial blood as the result of short periods of exercise.

The results of the exercise are given in Table I.

CO_2 -Combining Capacity.—As in normal men, there is always a lowering in the CO_2 absorption curve and a reduction of CO_2 capacity at 40 mm. of CO_2 tension. The greatest change was 11.5 volume per cent in William R. doing 2,185 kilogrammeters and in Chris Q. performing about 2,000 kilogrammeters, in 3 minutes. The change was marked in all cases, indicating that in diabetics as well as in normal men there is a utilization of available alkali through the formation of some acid in large amount.

CO₂ Tension.—There is as in the normal subjects a reduction in CO₂ tension, indicating a sufficiency of the respiratory mechanism not only to relieve the body of the great excess of CO₂ produced during the exercise but also to pump out some of the pre-formed CO₂ which has been liberated from the breakdown of bicarbonate. The one exception occurred in the first observation on William R., October 31, 1923, in whom the CO₂ tension was low, only 30 mm. during rest. 2 minutes after exercise it was somewhat higher than the resting value.

Blood Reaction.—The calculated reaction of the blood shows a constant reduction in pH, the greatest change being observed in George H., of pH 0.15 as the result of 2,004 kilogrammeters in 3½ minutes.

Qualitatively, the reaction of diabetics to exercise seems to be similar to the normal. There is in both a reduction in CO₂ capacity, CO₂ tension, and blood alkalinity. Quantitatively, it appears that for an equivalent amount of exercise the change both in CO₂ capacity and pH is greater in the diabetic.

The amount of work performed by the diabetics was much less than in the series of normal individuals. This was unavoidable as no one of the diabetics was able to continue at the rate of 1,000 kilogrammeters per minute. The difficulty seemed to be almost entirely in the muscles. The respirations were not labored at the end of exercise, the pulse rate was not unusually rapid, and there was no undue general fatigue; but the pain in the calf muscles of which so many diabetics complain became unbearable if a heavy weight was used or if a rapid rate was attempted. The same difficulty has been encountered in other pathologic conditions and was not considered specific to the diabetic state. The greatest amount of work was 2,355 kilogrammeters in 3¼ minutes, performed by William R. on December 1, 1923. In the series of normal individuals (5), D. P. B., who had an average response, did this work with only slight changes in acid-base equilibrium, 0.03 in pH and 3.3 volumes per cent in CO₂-combining capacity. With 1,300 kilogrammeters, the amount done by the diabetic Ben C., the change of D. P. B. was only 0.01 in pH and less than 1 volume per cent in CO₂ capacity.

Relation of Change in CO₂ Capacity to Lactic Acid and the Acetone Bodies.

A number of experiments were done to determine whether these changes could be attributed, as in normal individuals, to lactic acid accumulation or whether they were due to the formation of some other acid. In Table II are given the results of nine such experiments.

TABLE II.

Comparison of Changes in Lactic Acid and CO₂ Capacity Following Short Periods of Exercise.

Subject.	Date.	CO ₂ capacity at 40 mm. CO ₂ tension		Lactic acid content.		Observed difference of lactic acid.	Calculated* difference of lactic acid.
		Before.	After.	Before.	After.		
	1928	vol. per cent	vol. per cent	mg. per 100 cc.	mg. per 100 cc.	mg.	mg.
William R.	Oct. 31	37.0	27.5	21.7	38.5	16.8	38.0
	" 16	47.5	36.0	17.1	65.1	48.0	46.0
	Nov. 21	45.0	36.0	18.9	72.0	53.1	36.0
	Dec. 1	45.5	33.5	15.8	67.6	51.8	48.0
Chris. Q.	" 13	40.6	31.4	24.1	70.0	45.9	36.8
	" 22	42.0	33.5	9.5	46.6	37.1	34.0
	1928						
Jervis B.	Apr. 5	43.6	35.7	14.4	58.8	44.4	31.6
James F.	" 17	42.0	32.5	14.4	56.7	42.3	38.0
George H.	" 26	48.2	37.2	19.4	56.0	36.6	44.0
Total.....						376.0	352.4

* Calculation on the basis that 1 volume per cent of CO₂ is the stoichiometric equivalent of 4 mg. of lactic acid.

It will be seen that in every instance there is an increase in lactic acid after exercise, in one instance amounting to over 50 mg. per 100 cc. of blood. In the last two columns of the table a comparison is made between the amount of increase in lactic acid observed by analysis with that calculated from the change in CO₂ capacity on the basis that a reduction of 1 volume per cent CO₂ is stoichiometrically equivalent to the addition of 4 mg. of lactic acid. The agreement between the observed and calculated values is not

TABLE III.
Comparison of Changes in Lactic Acid and CO₂ Capacity Following Long Periods of Exercise.

Subject.	Date.	Condition.	Amount of work.	Duration of work.	CO ₂ capacity at 40 mm. CO ₂ tension.		Lactic acid content.		Observed difference of lactic acid.	Calculated* difference of lactic acid.
					Before.	After.	Before.	After.		
			kg. m.	min.	sol. per cent	sol. per cent	mg. per 100 cc.	mg. per 100 cc.	mg.	mg.
H. E. H.	1923	Normal.	14, 168	50	44.0	44.0	11.2	16.5	5.3	0.0
Morris G.	Feb. 19	Diabetic.	12, 054	50	45.0	34.5	16.1	52.9	36.8	42.0
James F.	" 15	"	10, 714	50	44.0	44.0	23.9	23.1	0.8	0.0
Hugh F.	Apr. 19	"	15, 642	50			17.5	27.7	10.2	
Irving R.	May 14	"	13, 540	50			20.8	33.3	12.5	
	" 16		14, 890	50	48.3	42.0	9.5	33.3	23.8	25.2

* Calculation on the basis that 1 volume per cent of CO₂ is the stoichiometric equivalent of 4 mg. of lactic acid.

exact, a discrepancy occurring in both directions. In seven of nine observations, the observed increase in lactic acid is greater than is necessary to account for the change in CO₂ capacity. The discrepancies are no greater than in the series of normal individuals. As will be seen in the table, the totals of observed and calculated differences are in fairly close agreement, the values being 376 mg. for observed and 352 mg. for calculated. The results seem to

TABLE IV.
Comparison of Total Acetone in Blood Before and After Exercise.

Subject.	Date.	Amount of work.	Duration of work.	Source of sample.	Total acetone.	
					Before.	After.
	1922	kg.m.	min.		mg. per 100 cc.	mg. per 100 cc.
William R.	Nov. 10	2,064	4	Artery.	17.8	18.8
	" 16	2,185	3	"	20.2	20.2
				Vein.		20.0
	Nov. 21	2,240	3½	Artery.	8.4	14.1
	Dec. 1	2,355	3½	"	4.0—*	4.0—
				Vein.		4.0—
Morris C.	Nov. 24	1,708	3½	Artery.	15.3	21.8
Chris. Q.	Dec. 13	2,000?	3½?	"	32.8	28.1
	" 22	2,000?	3½?	"	4.0—	4.0—
				Vein.		4.0—
	1923					
Jervis B.	Apr. 5	1,500?	3½?	Artery.	4.0—	4.0—
	" 10	1,500?	3½?	"	6.3	7.5
James F.	" 17	2,000?	3½?	"	18.9	15.8
George H.	" 26	2,004	3½	"	29.0	22.5
Morris G.	Feb. 15	12,054	50	Vein.	15.6	22.0
James F.	Apr. 19	10,714	50	"	4.0—	4.0—
Hugh F.	May 14	15,642	50	"	24.2	21.4
Irving R.	" 16	13,540	50	"	35.0	36.0
	" 25	14,890	50	"	23.4	19.0

*4.0 — indicates 4.0 mg. or less.

indicate that the changes observed in CO₂ capacity are accounted for quantitatively by the accumulation of lactic acid. In these observations it is unnecessary to search for or assume any other acid as the cause for the disturbance of acid-base equilibrium.

Besides the observations on short periods of exercise, several experiments were done in which the work was less severe but more

prolonged. The duration was in all instances 50 minutes, the amount varying from 234 to 313 kilogrammeters per minute. This is exercise which a normal individual may do without demonstrable change in CO_2 capacity and without appreciable lactic acid accumulation. This was instanced by an experiment on H. E. H., a normal man, whose reaction to exercise has been extensively studied. In this the CO_2 capacity of the venous blood was unchanged and there was an increase of only 5.3 mg. of lactic acid in the venous blood. Five observations on four diabetics are reported in Table III. One of them, James F., April 19, 1923, had the reaction of a normal individual with no change in CO_2 capacity and with no demonstrable change in lactic acid. All the others had an increase in lactic acid. In those upon whose blood CO_2 analyses were done the increase approximates closely that calculated from the reduction in CO_2 capacity.

In both short and long periods of exercise determination of total acetone bodies in the blood was made before and after the exertion. The results are presented in Table IV. It will be seen that there is a slight variation in both directions. The maximum changes are $+ 6.5$ and $- 6.4$, following the exercise. As the error in the acetone determination is ± 3 mg. per 100 cc. these changes are not significant. The observations fail to show any appreciable accumulation of acetone bodies in the blood.

Disappearance of Lactic Acid.

Several experiments were performed to discover whether the disappearance of lactic acid in the tissues of the arm could be demonstrated after leg exercise. The results of these will be found in Table V. Samples of blood were taken simultaneously from the brachial artery and median basilic vein from 2 to 4 minutes after the exercise unless otherwise specified in the table. In all the observations, there is a difference between arterial and venous blood. The CO_2 -combining capacity is always higher and the lactic acid content with one exception is lower in the venous blood. The greatest difference in CO_2 capacity is 5.1 volumes per cent in William R., December 1, 1923. The greatest loss of lactic acid is observed in Chris Q., December 13, 1923, in

TABLE V.
Comparison of Arterial and Venous Blood from the Arm Following Short Periods of Leg Exercise.

Subject.	Date.	CO ₂ capacity at 40 mm. CO ₂ tension.			Lactic acid content.				Remarks.
		Arterial.	Venous.	Differ- ence.	Arterial.	Venous.	Observed difference.	Calcu- lated difference.	
William R.	1949								
	Oct. 31	27.5	30.0	+2.5	38.5	46.9	+8.4	-10.0	Simultaneous samples 2 to 4 min. after exercise.
	Nov. 16	36.0			65.1	43.8	-21.3		"
	Dec. 1	33.5	38.6	+5.1	67.6	49.7	-17.9	-20.4	"
Chris. Q.	" 13	31.4			70.0	50.3	-19.7		"
	" 22	33.5	36.3	+2.8	46.6	38.5	-8.1	-11.2	"
Ben C.	1948								
	Feb. 3	36.3	41.2	+4.9	69.0	62.0	-7.0	-19.6	Arterial sample 6 to 8 min. after. Venous sample 2 to 3 min. after.

whom the venous blood contained 20 mg. less than the arterial. In the observation on William R., the lactic acid content is lower in the arterial than in the venous blood, although the CO₂ capacity determinations indicate a loss of acid during the passage of blood through the tissues of the arm. No positive source of error could be demonstrated in the lactic acid determinations, but it is worthy of note that the acetone corrections were much higher in the arterial blood than in any other estimation which we have made. In the venous blood the acetone correction was no greater than usual. In the experiment on Ben C., the samples were not simultaneous. It is interesting that the lactic acid content of the venous blood taken 2 to 3 minutes after exertion is lower than the content of arterial blood drawn 7 minutes after the exercise.

DISCUSSION.

In the diabetics which have been observed in this study, the results give no hint that during exercise the body is unable to produce lactic acid or to dispose of it after it is formed. There is no indication that the chemical mechanism of muscular contraction need be anything else than the breakdown of carbohydrate to lactic acid which is utilized in muscular work in normal man. The experiments demonstrate clearly that in the moderately severe diabetic individual, short periods of exercise lead to the formation of lactic acid in large amounts and do not cause accumulation of acetone bodies in the circulating blood. From the theoretic standpoint, however, the experiments must of necessity be inconclusive. All the patients were capable of utilizing some carbohydrate. It is entirely possible that the individuals whom we studied may have retained enough of the normal functions of carbohydrate utilization to perform the muscular contractions which were required of them by the usual mechanism and for that reason exhibited the normal accumulation of lactic acid. A different mechanism might conceivably be employed in completely diabetic patients. In the human subject with complete diabetes, exercise sufficiently great to cause accumulation of lactic acid in the blood, is not feasible because of the serious condition of the patients. Experiments upon lactic acid and acetone production.

during exercise of completely diabetic animals are now in progress and will be reported in a subsequent paper.

Only hints concerning the effect of exercise upon the well-being of diabetic patients can be obtained from the foregoing observations. The two kinds of exercise which were studied, were such as might be used by any ambulant diabetic during the course of his daily life routine. The short periods of exercise correspond to moderately rapid stair climbing or a short run at a dog trot. The work of longer duration roughly approximates 50 minutes of rather rapid walking. No attempt was made to carry the exertion to the point of fatigue. The experiments indicate with both kinds of exercise a marked acid production and a considerable reduction in the alkaline reserve. In none of our cases was it sufficient to cause any alarming depletion of the available alkali. Unfortunately, however, none of our patients had a particularly low alkaline reserve before the exercise was started. The observations do not exclude the possibility of serious damage from exercise in a diabetic with initial severe acidosis.

CONCLUSIONS.

1. Following short periods of vigorous exercise in the diabetic, there are changes in acid-base equilibrium somewhat greater than in normal individuals doing a comparable amount of work. In the arterial blood, these are apparent in a reduction of CO_2 capacity, CO_2 tension, and alkalinity.

2. There is a marked increase of lactic acid in the circulating blood, the amount varying in the different individuals from 16.8 to 53.1 mg. per 100 cc. This is also apparent following more moderate exercise of 50 minutes duration.

3. The reduction in CO_2 capacity can be accounted for quantitatively by the increase in lactic acid in the diabetics whom we have studied.

4. No significant change in the concentration of acetone bodies in the blood can be demonstrated as a result of exercise for short periods. During 50 minutes of more moderate exercise, there is no accumulation of acetone.

5. Following leg exercise, a study of simultaneous samples of arterial and venous blood from the arm reveals in diabetics as in

normal individuals a disappearance of lactic acid and a rise in CO_2 capacity during the passage of blood through the tissues of the arm. The loss of lactic acid varied between 8.1 and 21.3 mg. in the different subjects.

6. The accumulation of lactic acid in the blood after exercise indicates that in the diabetic individuals reported in this paper, the breakdown of carbohydrate to lactic acid was employed as the chemical mechanism of muscular contraction.

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Case Histories.

Case 1.—William R. Moderate diabetes. Age 29 years. Grocery clerk. First symptoms in July, 1922, when sugar was found in urine. Lost 40 lbs. in weight from July to Oct., 1922. Admitted to medical service on Oct. 2, with sugar ++++ and a trace of diacetic acid in his urine. Blood sugar was 464 mg. on Oct. 4 and 420 mg. on Oct. 16. Oct. 30. Diet supposedly C 15, P 15, and F 90;³ but patient undoubtedly was breaking diet. 24 hour urine contained sugar 28.0 gm. Reaction to exercise studied Oct. 31. Nov. 5. Transferred to metabolism ward where conditions were carefully controlled. Diet from Nov. 5 to 10. C 15, P 15, and F 90. Sugar in 24 hour urine fell from 96.5 gm. on Nov. 6 to a trace on Nov. 10. Total acetone in urine fell from 8.8 gm. on Nov. 6 to 3.2 gm. on Nov. 10. Reaction to exercise studied Nov. 10. Nov. 15. Diet changed to C 20, P 20, and F 100. No sugar in urine. 24 hour specimen contained 3.41 gm. of total acetone. Reaction to exercise studied Nov. 16. Nov. 20. Diet C 20, P 50, and F 100. No sugar in urine. Total acetone 1.71 gm. in 24 hours. Reaction to exercise studied Nov. 21. Beginning Nov. 22 the diet was gradually increased until on Nov. 28 he was receiving C 85, P 50, and F 100. On the following day he showed a trace of sugar. Nov. 30. Diet C 80, P 50, and F 100. No sugar in urine. 24 hour specimen contained 0.30 gm. of total acetone. Reaction to exercise studied Dec. 1.

Case 2.—Jervis B.⁴ Severe diabetes. Age 26 years. Storekeeper. Duration of symptoms 1 year. Sugar was found in urine early. Diet had been fairly strict at times during year preceding admission. Had lost 25 lbs. in weight. Had drowsiness, vomiting, and epigastric pain for 1 week before admission. Admitted Mar. 9, 1923. He was drowsy and hard to arouse; had hyperpnea with slow, deep respirations. Urine sugar ++++, acetone and diacetic acid tests positive. Blood sugar 410 mg. on admission. Was placed on diet of milk and glucose. On Mar. 12, blood sugar 500 mg., CO₂ capacity of plasma, 30 vols. per cent. Mar. 13. Admitted to metabolism ward. Blood sugar 326 mg., total acetone 54. 24 hour urine contained 18.3 gm. of sugar and 8.0 gm. of total acetone. From Mar. 13 to 18 he was fasted. On the 3rd day of fasting his blood sugar was 157 mg., total acetone 61 mg., urine contained 11.1 gm. of glucose and 9.1 gm. of total acetone. At end of fast there were 4.4 gm. of glucose and 7.6 gm. of total acetone in urine. He was then placed on iletin and a diet of C 50, P 50, and F 50 upon which he became sugar-free on Mar. 23. Apr. 5. Diet C 100, P 70, and F 150 which he had been taking with iletin since Apr. 1. Urine contained 22.8 gm. of sugar and 0.45 gm. of total acetone. Reaction to exercise was studied. Apr. 10. Diet C 40, P 70, and F 150. Urine con-

³ Throughout the case histories the diet will be given in terms of C designating carbohydrate in grams; P, protein in grams; and F, fat in grams.

⁴ Calorimeter studies on Cases 2 to 6 inclusive can be found in an article by Richardson and Ladd (29).

tained 12.4 gm. of sugar in 24 hours. Reaction to exercise studied. No insulin was being administered at this time.

Case 3.—Frank C. Moderate diabetes. Age 28 years. Accountant. First symptoms Jan., 1921. Had had frequent attacks of "sore eye," probably iritis, for 2 years preceding the onset. Studied in metabolism ward in Feb., 1922, when blood sugar was 210 mg. per 100 cc. CO₂ capacity of plasma was normal. Became sugar-free without fasting. In June, 1922, developed jaundice during which his tolerance fell to a low level. Readmitted Nov. 10 suffering from a balanitis. The liver was slightly enlarged and the knee-jerks were absent. There was much sugar and acetone in the urine. Became sugar-free after 1 day of a diet of C 15, P 15, and F 90. Diet gradually increased. Nov. 19. Diet of C 30, P 40, and F 170 caused glycosuria. Dec. 7. On diet of C 58, P 36, and F 53, lost 6.5 gm. of glucose in the urine. Acetonuria was persistent. On Nov. 23, 1 day before the exercise experiment, his blood sugar was 171 mg. per 100 cc. CO₂ capacity of plasma 69 vols. per cent. Urine was sugar-free and contained 0.26 gm. of acetone on a diet of C 40, P 26, and F 38.

Case 4.—Morris G. Moderately severe diabetes. Age 25 years. Roumanian. First symptoms in Dec., 1920. Had lost 23 lbs. in 2 years. Admitted to metabolism ward on Jan. 9, 1923. During a period of more than a month he was unable to tolerate without insulin a diet greater than the lowest Newburgh diet of 14 gm. of carbohydrate and 935 calories. Greatest excretion of acetone bodies was 2.24 gm. on a balanced diet. On Feb. 14, the day before the exercise experiment, he excreted 14 gm. of sugar and 2.01 gm. of acetone. He was not on insulin at the time of the experiment.

Case 5.—George H. Moderate diabetes. Age 25 years. Clerk. Born in the United States. Was admitted to metabolism ward on Apr. 17, 1923. Onset of symptoms in Dec., 1922. He was observed until Apr. 28. During this period he was sugar-free on diets varying from C 5, P 14, and F 30 to C 30, P 40, and F 180. Acetone excretion varied between 0.8 and 2.5 gm. per 24 hours. Reaction to exercise was studied on Apr. 26 when urine was sugar-free but contained 2.5 gm. of acetone per 24 hours on a diet of C 30, P 40, and F 165.

Case 6.—Chris Q. Mild diabetes. Age 35 years. Fireman. Born in the United States. Was admitted to metabolism ward on Dec. 23, 1922. The symptoms of diabetes dated back 8 months. His blood sugar varied between 227 and 126 mg. per 100 cc. The maximum excretion of acetone bodies was 2.01 gm. on a high fat diet. The lowest CO₂-combining power of the blood was 54 vols. per cent. He developed a tolerance of 50 gm. of C while in the ward. On Dec. 12, the day before the first exercise experiment, urine was sugar-free and contained 2.09 gm. of acetone on a diet of C 15, P 15, and F 90. Blood sugar was 208 mg. per 100 cc. On Dec. 21, the

day before the second experiment, the urine was negative for sugar and contained 0.21 gm. of acetone on a diet of C 80, P 70, and F 100.

Case 7.—Ben C. Severe diabetes. Age 25 years. Violinist. Was admitted to metabolism ward on Jan. 17, 1923. Mother has diabetes and sister died of it. He had been under treatment for 3 years and recently had required the use of insulin. On a high fat diet with insulin he excreted a maximum of 3.0 gm. of acetone bodies in 24 hours. When insulin was discontinued, the glucose in the urine reached 54 gm. He failed to develop any spontaneous tolerance for carbohydrate while in the ward. From Jan. 31 to Feb. 3 his diet was C 30, P 50, and F 100 with 12 units of iletin three times a day. At this time he had no glycosuria and only 0.5 mg. of total acetone in urine. Reaction to exercise was studied on Feb. 3. Attempt to study exercise while patient was without iletin was unsuccessful.

Case 8.—Hugh F. Moderately severe diabetes. Age 25 years. First symptoms were noted about Aug., 1922. At time of admission, Apr. 23, 1923, he was drowsy. Urine contained much sugar, acetone, and diacetic acid. On Apr. 27, after 4 days of treatment, blood sugar was 400 mg. per 100 cc. On May 6, the day before the first experiment, he had 31 gm. of sugar in the urine, but no diacetic acid or acetone on a diet of C 40, P 40, and F 100. On May 17, the day before the last experiment, there was no glycosuria or acetonuria on a diet of C 30, P 70, and F 80.

Case 9.—Morris C. Severe diabetes? Age 40 years. Had sugar in his urine for 3 years before admission. Was admitted to general medical service on Apr. 19, 1923 with acetone in urine and breath and a large amount of sugar in his urine. Blood sugar was 395 mg. per 100 cc. He showed sugar for a long period on a diet of C 15, P 15, and F 90 but several times was found to be breaking diet. On May 23, his reaction to exercise was studied. At that time his blood contained 500 mg. of sugar.

Case 10.—Irving R. Moderate diabetes. Age 23 years. First symptoms were observed in 1921. During the 2 year period he had practiced dietary restriction with varying fidelity. Lost about 25 lbs. in weight. He was admitted on May 11, 1923. He was fasted until May 14 when his urine contained 1.3 gm. of sugar. Blood sugar was 210 mg. CO₂ capacity of plasma 54 vols. per cent. His reaction to exercise was studied May 16. On May 24, he excreted 19.0 gm. of sugar on a diet of C 35, P 80, and F 125. Exercise experiment was performed on May 25 at which time his blood contained 235 mg. of sugar per 100 cc.

Case 11.—James F. Moderate diabetes. Age 28 years. First admitted to hospital in Dec., 1921. Comatose from carbon monoxide poisoning following an alcoholic debauch. Sugar was found in his urine. 1 week later he developed scarlet fever. Second admission Feb. 25, 1922 with veronal poisoning. He was drowsy. Tongue red and dry. Moderate hyperpnea, acetone in breath and urine. Urinary sugar + + +. Admitted

for the third time on Apr. 10, 1923 because of polyuria and weakness—having had several crops of boils on the buttocks. Drowsy. The rate and depth of respirations were moderately increased. Urine contained much sugar, but no acetone. Blood sugar Apr. 11 was 360 mg. That day he entered the metabolism ward and was put on a diet of C 34.0, P 34.1, and F 34.2. On Apr. 16, 1 day before an experiment, he was sugar-free for the first time, on a diet of C 14.0, P 39.9, and F 90.0. Urinary acetone was 2.06 gm. On Apr. 18, his urine revealed a trace of sugar and 0.23 gm. of ketone on a diet of C 35.0, P 75.0, and F 100.0. An experiment was performed the next day. „

Protocols.

Subject.	Date.	Source of blood specimen and time taken.	CO ₂ absorption curve.*		CO ₂ content of blood of blood as drawn.	O ₂ content of blood as drawn.	O ₂ capacity.	Lactic acid.	Total acetone.	Blood sugar.	Remarks.
			CO ₂ tension.	CO ₂ content.							
William R.	1922 Oct. 31	Arterial blood before exercise.	mm.	vol. per cent.	vol. per cent.	vol. per cent.	vol. per cent.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	Exercise = 1,764 kg.m. in 3½ min.
			40.3	36.7	33.3	17.3	18.4	22.4			
			69.5	36.9	33.5	17.6	18.2	21.0			
				45.1							
				44.6							
		Arterial blood after exercise.	34.5	25.0	25.7	19.3	20.7	39.2			Exercise = 2,064 kg.m. in 4 min.
			56.2	25.3	25.6	19.0		37.8			
				34.2							
				34.7							
			32.2	26.7	28.5	17.2	20.6	48.3			
	Nov. 10	Venous blood after exercise.		28.9	17.0	20.5		45.5			Exercise = 2,064 kg.m. in 4 min.
									17.8		
			41.0	44.0				24.5			
				43.8				23.1			
			42.2	42.0				31.5	18.8		
		Arterial blood after exercise.		41.5				31.5			Exercise = 2,185 kg.m. in 3 min.
			32.4	44.7	48.8	19.1	20.6	17.1	20.2	151	
			62.9	54.9	48.8	19.5		17.1			

Nov. 21	Arterial blood after exercise.	36.0	33.8 34.3	35.0 34.8	21.2 21.0	21.4 21.5	65.1 65.1	20.2	162
	Venous blood after exercise.			46.7 46.3	14.6 14.9		42.7 44.8	20.0	138
	Arterial blood before exercise.	31.2 63.6	42.1 42.1 54.4	47.8 47.5	18.3 17.9		19.6 18.2	7.5 9.3	153
	Arterial blood after exercise.	30.9 43.2	31.3 37.6 38.0	35.4 34.5	20.1 20.7	22.4	72.9 71.1	16.9 11.2	220
Dec. 1	Venous blood after exercise.						32.8 30.9		148
	Arterial blood before exercise.	30.9	41.5 42.4	47.9 48.1	16.9 16.4	16.8	15.8		142
	Arterial blood after exercise.	29.7	28.4 28.5	32.9 32.9	18.2 18.0	18.4	67.6		143
	Venous blood after exercise.	30.2	33.8 33.4	44.7 44.5	13.0 12.3		49.7		144

Exercise = 2,240 kg.m.
in 3½ min.

Exercise = 2,355 kg.m.
in 3½ min. For calculation of CO₂ tension and pH use slopes of curves obtained on Nov. 21.

* If the absorption curves be plotted, it will be noticed that the slope of the curve is in all instances steeper after exercise than before. If the formula of Van Slyke for buffer values $\frac{[\text{ABHCO}_3]}{\Delta\text{pH}}$ 30-60, is used, it will be seen that the buffer value is higher after exercise. This was a matter of surprise to us since previous work with normal individuals gave exactly opposite results, indicating a flatter absorption curve and a lower buffer value following exercise. Although the indicated increase in buffer value is consistent in all experiments, and in some not here published, we are unwilling at present to attach any significance to the finding or to draw any conclusion from two-point absorption curves.

Subject.	Date.	Source of blood specimen and time taken.	CO ₂ absorption curve.		CO ₂ content of blood as drawn.	O ₂ content of blood as drawn.	O ₂ capacity.	Lactic acid.	Total acetone.	Blood sugar.	Remarks.
			CO ₂ tension.	CO ₂ content.							
			mm. Hg	vol. per cent	vol. per cent	vol. per cent	vol. per cent	mg. per 100 cc.	mg. per 100 cc.		
Frank C.	1922 Nov. 24	Venous blood before exercise.	54.9	53.9				23.1	15.3		Exercise = 1,708 kg.m. in 3½ min.
		Venous blood after exercise.	39.5	44.2				35.7	21.8		
Chris Q.	Dec. 13	Arterial blood before exercise.	35.9 47.0	39.1 43.1	43.1 43.1	21.6 21.2	21.9	25.2 23.1	32.8	121	Amount of work not recorded. Approximately 2,000 kg.m. in 3½ min. Arterial and venous samples taken simultaneously 2 to 4 min. after exercise.
		Arterial blood after exercise.	28.4 42.6	26.0 32.8	28.9 29.3	23.0 23.0	24.4	68.6 71.4	28.1	134	
		Venous blood after exercise.						51.5 49.0		124	
	Dec. 22	Arterial blood before exercise.	31.3 74.6	38.9 54.7	45.0 44.1	19.3 18.9		9.5	Less than 4 mg.	115	Amount of work not recorded. Approximately 2,000 kg.m. in 3½ min. Arterial and venous samples taken simultaneously 2 to 4 min. after exercise.
Arterial blood after exercise.		30.5 49.4	29.7 36.8	33.5 34.0	22.0 21.7	46.6	Less than 4 mg.	112			

Ben C.	1923 Feb. 3	Venous blood after exercise.	30.6	32.6 32.5	48.8 49.1	6.9? 5.1?		38.5	Less than 4 mg.	113	
		Arterial blood be- fore exercise.	30.4 63.3	45.0 45.0 58.2	50.0 50.1	18.1 18.3	18.2			126	Exercise = 1,386 kg.m. in 3 min. Venous sample taken 2 to 3 min. after exercise.
		Arterial blood after exercise.	30.1	32.1	36.1	19.5		69.0		117	Arterial blood 6 to 8 min. after exercise.
Morris G.	Feb. 15	Venous blood after exercise.	30.6 62.9	37.2 51.6	54.7	7.0	19.6	62.0		120	O ₂ capacity by Lunds- gaard technique.
		Venous blood be- fore exercise.	50.3	48.9				16.1	15.6	240	Exercise = 12,054 kg.m. in 50 min.
		Venous blood after exercise.	18.2 50.5	24.2 24.5 38.1 39.0				52.9	22.0	244	
Jervis B.	Apr. 5	Arterial blood be- fore exercise.	26.0	38.5	48.1 48.5	18.7 18.5	18.9 18.7	14.7 14.0	Less than 4 mg.	138	Exercise not recorded. Approximately 1,500 kg.m. in 3½ min. Slope of curves Apr. 10, 1923.

George H.	Apr. 19	Venous blood before exercise.	29.0 57.9	39.2 53.1 53.4				24.3 23.4	Less than 4 mg.	167	Exercise = 10,714 kg.m. in 50 min.
		Venous blood after exercise.	29.1 53.9	37.6 37.4 52.2				22.4 23.8	Less than 4 mg.	165	
	Apr. 26	Arterial blood before exercise.	29.5 53.9	43.8 53.8 54.2	47.4 47.9	19.4 19.5	18.9 19.0	19.2 19.6	29.0	158	Exercise = 2,004 kg.m. in 3½ min.
		Arterial blood after exercise.	29.7 49.6	32.1 32.3 41.5 41.4	34.4 34.8	20.3 20.1	20.2 20.2	53.2 58.8	22.5		O ₂ capacity by Lunds-gaard technique.
Hugh F.	May 7	Venous blood resting.	16.0 28.7 53.3	32.9 32.1 40.3 50.4						375	
	May 8	Venous blood after exercise.	13.5 26.8 53.1 69.0	20.6 20.5 28.1 39.6 46.7 46.9				69.3 65.8		500	Exercise = 3,071 kg.m. in 3½ min.

Subject.	Date.	Source of blood specimen and time taken.	CO ₂ absorption curve.		CO ₂ content of blood as drawn.	O ₂ content of blood as drawn.	O ₂ capacity.	Lactic acid.	Total acetone.	Blood sugar.	Remarks.
			CO ₂ tension.	CO ₂ content.							
	1923		mm. Hg	vol. per cent	vol. per cent	vol. per cent	vol. per cent	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	
Hugh F.	May 14	Venous blood before exercise.						18.8	24.2	146	Exercise = 15,462 kg.m. in 50 min.
		Venous blood after exercise.						16.1	21.4	167	
Irving R.	May 16	Venous blood before exercise.						25.2	35.0	246	Exercise = 13,540 kg.m. in 50 min.
		Venous blood after exercise.						30.1	36.0	244	
	May 25	Venous blood before exercise.	40.8	48.5				9.8	23.4	231	Exercise = 14,890 kg.m. in 50 min.
		Venous blood after exercise.	39.7	41.8				9.1	19.0	190	
H. E. H.	Feb. 19	Venous blood before exercise.	19.2	33.9				32.9			Normal man. Exercise = 14,168 kg.m. in 50 min.
		Venous blood after exercise.	55.2	50.7				33.6			
				51.2				11.2			

D. P. B.	Venous blood after exercise.	19.6	33.5	16.5	Experiments as check on technique. Absorp- tion curve of same in- dividual on 2 succes- sive days.	
		55.9	33.7			
			51.6			
			50.6			
	Mar. 30	Venous blood resting.	19.0	31.7		
			44.9	32.1		
				46.4		
			69.4	46.3		
	Mar. 31	Venous blood resting.		55.7		
				55.5		
			19.3	33.4		
			45.3	33.1		
			48.7			
		70.0	48.4			
			57.1			
			57.0			

STUDIES IN THE CHEMISTRY OF HEMOGLOBIN.

II. A METHOD FOR THE STUDY OF THE EQUILIBRIUM BETWEEN OXYGEN AND HEMOGLOBIN.

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INTRODUCTION.

The equilibrium between oxygen and hemoglobin is of unquestioned importance, both from a physiological and from a purely chemical standpoint. Indeed, this is so well recognized that a tremendous amount of investigation has for the last 60 years been devoted to this subject.

Physiologically not only does hemoglobin transport oxygen but it also plays an important rôle in the carriage of carbon dioxide; in the acid-base equilibrium in the blood; in the partition of chlorides and of water between the red blood cells and plasma; and also, no doubt, in various other phenomena which are less clearly understood. These various processes are known to depend in greater or less degree upon the equilibrium between oxygen and hemoglobin.

Chemically the problem is of no less interest. Today, after a considerable period of uncertainty, we can feel virtually certain that the combination of hemoglobin and oxygen is chemical. But, despite many attempts, a quantitative description and interpretation of the process remains to be given. Even the nature of the union is uncertain, although the present indications are that hemoglobin does not carry oxygen in a manner that is usual in organic chemistry. Thus it appears to be a rather unique substance. It is now evident that several interdependent equilibria are simultaneously involved in solutions containing

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oxygen and hemoglobin: the acid-base equilibria of reduced and oxyhemoglobin; the equilibrium between met- and reduced hemoglobin; and, in particular, the equilibrium between oxygen and hemoglobin. Since all are undoubtedly of considerable importance, we expect to study them in the near future. In this paper, however, we are concerned exclusively with a method for the study of the equilibrium between oxygen and hemoglobin.

Historical.

Several methods have been described heretofore. We shall attempt merely to outline them.

The earlier workers depended on the use of the gas pump. A satisfactory description of this method is given by Bohr (1). In such work a sample of hemoglobin was exposed to a given tension of oxygen. A portion of this sample was then connected with a gas pump of convenient design and the contained gas (or gases) pumped off, analyzed, and measured. Such a method was necessarily slow, cumbersome, and, as usually employed, of a relatively low order of accuracy.

Certain later workers made use of indirect spectrophotometric methods. These involved the use of diluted samples of hemoglobin and necessitated calibration by more direct physical or chemical methods.

The discovery by Haldane (2) that ferricyanide liberates oxygen from hemoglobin enabled Barcroft (3) to develop his "differential blood gas apparatus." In this method two chambers provided with suitable stop-cocks are both connected with a U-tube manometer containing clove oil. Consequently, when both chambers are closed to the outside air, any increase in the volume of gas contained in one chamber will result in a displacement of the column of clove oil, which is proportional to the increased amount of gas. Hemoglobin which had previously been equilibrated with a given oxygen tension was placed in both chambers of such an apparatus. Potassium ferricyanide was added to the solution in one chamber, thereby releasing the oxygen chemically bound; the quantity could be calculated from the differences in level in the manometer. Barcroft (4) indicates that the experimental error is of the order of ± 1.0 volume per cent. Since we wish an exact quantitative method this degree of accuracy is insufficient. In addition, it has the further objections, in common with all methods where ferricyanide is used, that samples must be used and transferred, and that the oxyhemoglobin is inevitably changed by the addition of this foreign substance; it cannot utilize the reversible reaction between oxygen and hemoglobin by working with a single sample of hemoglobin exposed successively to varying tensions of oxygen and cannot, therefore, be made continuous.

Van Slyke (5) in his apparatus combined the chemical principle of liberating oxygen by means of ferricyanide with the physical method of extraction by a partial vacuum. His last described constant volume apparatus (6) is the more accurate, the principle remaining the same. Hemoglobin, previously equilibrated in a suitable container, is admitted into a chamber over mercury, ferricyanide added, and the oxygen extracted by means of a torricellian vacuum; the gas is then measured in the same chamber in which it is liberated. Although the accuracy of about 0.1 volume per cent claimed for this apparatus is a real improvement, its use is still open to the objections that samples must be used and transferred, that ferricyanide destroys the further usefulness of the same hemoglobin, and that this process also cannot be made continuous.

Professor Krogh has recently called to our attention the fact that Bohr (7) had long since developed an apparatus similar in principle to one developed independently by us. Bohr's apparatus consisted essentially in a chamber of known volume half filled with a known volume of hemoglobin solution connected by means of glass tubing and a stop-cock with a gas burette. A given amount of oxygen was introduced into the burette, measured, the stop-cock into the hemoglobin chamber opened, and the whole apparatus shaken. Subsequently the pressure of the oxygen was determined. Since the volume relationships of the gas and liquid phases were known, the amount of gas in the solution could readily be calculated.

Now, whereas Bohr's method is sound in theory, in analyses by him the order of accuracy attained was probably about ± 0.5 volume per cent.

We have, therefore, sought to develop a method which like Bohr's method—a procedure which was unknown to us at the time this investigation was undertaken—depended chiefly on physical means, which left the hemoglobin as nearly unchanged as possible and could, therefore, take advantage of the reversible equilibrium between oxygen and hemoglobin but which was susceptible of considerable accuracy.

General Description of the Method.

To attain this end we have devised an apparatus (Fig. 1) of known volume in which a measured volume of hemoglobin was brought into equilibrium with varying quantities of oxygen. Since our apparatus was so arranged that we could remove a portion of the gas phase without altering equilibrium in the liquid phase, we could measure this fraction, subsequently calculating not only the amount of oxygen in the entire gas phase but also, through application of the gas laws, its tension. The difference between the quantity of oxygen introduced into the

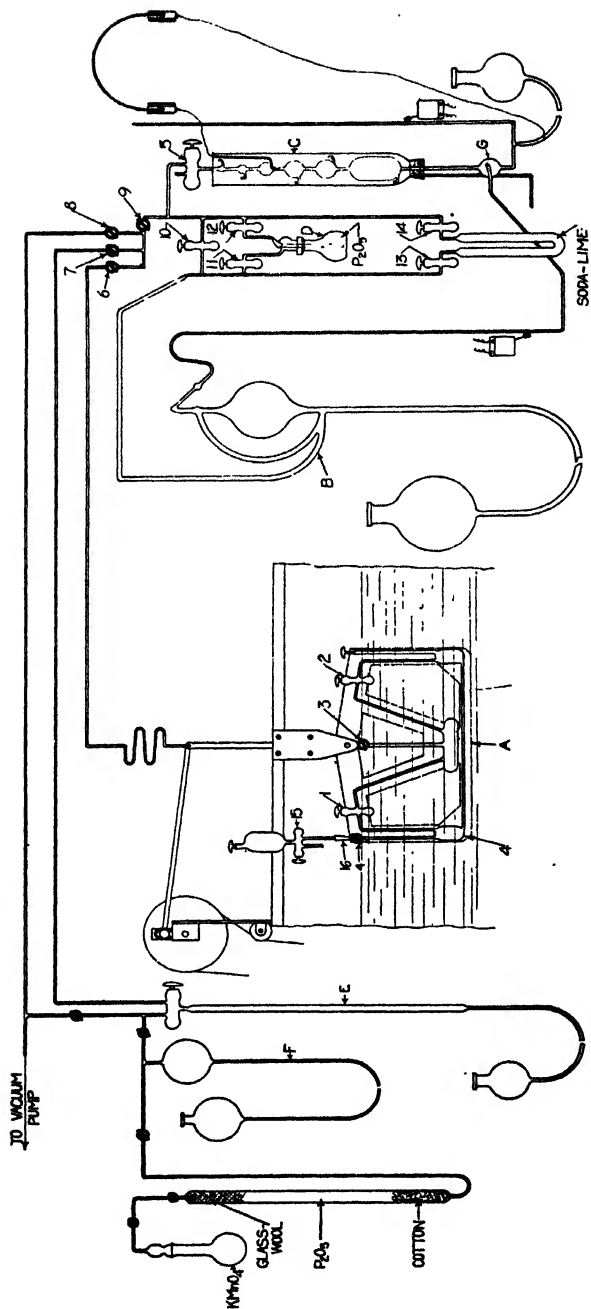


Fig. 1.

apparatus and that in the gas phase represented the amount taken up in the liquid phase, from this the amount in combination with hemoglobin could be calculated. As designed, the apparatus consisted of the equilibrator (A); a mercury pump for transferring gas from a large portion of the gas phase; suitable devices for absorbing water vapor and other gases; a specially designed burette (C) and manometer for the accurate measurement of gases; a mercury distillation pump of the Langmuir type for the removal of gases from the apparatus; a suitable device for the preparation and storage of pure oxygen; a cathetometer for the accurate reading of pressure; and suitable glass connections. It is to be noted that the entire apparatus was a closed system and was built entirely of glass.

Detailed Description of Apparatus.

Gas Burette.

1. *Description.*—Since the gas burette was used in calibrating other portions of the apparatus it is desirable to describe it first. It was somewhat similar to the U. S. Bureau of Mines type of gas burette and consisted of a series of six superimposed glass chambers of sizes varying from approximately 1.0 to 63.0 cc. in capacity.

Above the smallest, a 3-way oblique stop-cock was sealed on, so that the two outlets were directed upwards. After calibration one end was closed, the other sealed into the system. The chambers were connected by short lengths of capillary tubing of uniform size. At the point where each chamber merged into the capillary, a finely tapered platinum point was sealed into the glass in such a manner that the tip was well in the center of the lumen of the capillary. This arrangement is similar to one used by Richards and Sameshima in compressibility studies (8). Since the capillary tubing between the burette chambers and in the connecting manometer had the same internal diameter, placing the points in this way served to minimize differences in capillarity between the burette and manometer. It is to be noted that the platinum points were carefully ground and examined under a hand lens in order to insure a taper to a true point.

In order that an electric connection could be made with each point, a short side arm of glass tubing was sealed on and bent upwards at the point where the blunt end of the platinum wire emerged from the burette. The side arms were connected with pieces of glass tubing which were car-

TABLE I.
Calibration of Burette.

Date.	Weight of Hg.	t Burette.	Weight in vacuo.	ΔHg	Volume No.	Volume.	Remarks.
	gm.		gm.			cc.	
Aug. 1, 1920	17.810	20.95	17.809	13.5439	1	1.315	
	17.827	20.92	17.826	13.5439	1	1.316	
	17.794	20.95	17.793	13.5439	1	1.314	
Jan. 11, 1923	17.809	21.5	17.808	13.5425	1	1.315	
Mean.....						1.315	Maximum deviation ±0.001
Apr. 1, 1920	23.127	20.1	23.126	13.5460	2	1.707	Original value.
Jan. 11, 1923	23.118	20.4	23.117	13.5452	2	1.707	"
	23.137	21.2	23.136	13.5433	2	1.708	After repairs.
	23.106	21.5	23.105	13.5424	2	1.706	
Mean.....						1.707	Maximum deviation ±0.001
Jan. 11, 1923	118.799	21.2	118.793	13.5433	3	8.771	
Jan. 12, 1923	118.828	21.2	118.822	13.5433	3	8.773	
	118.809	21.4	118.803	13.5428	3	8.772	
	118.833	20.5	118.827	13.5449	3	8.773	
Mean.....						8.772	Maximum deviation ±0.001
Jan. 4, 1923	150.379	21.2	150.371	13.5433	4	11.103	
" 12, 1923	150.373	21.4	150.366	13.5428	4	11.103	
Mean.....						11.103	Maximum deviation ±0.000

Mar. 22, 1920	357.13	21.7	357.11	13.5421	5	26.370	Maximum deviation ± 0.0005
" 30, 1920	357.14	21.7	357.12	13.5421	5	26.371	
" 31, 1920	357.13	21.8	357.11	13.5418	5	26.370	
Mean.....						26.370
Mar. 22, 1923	857.84	24.0	857.80	13.5365	6	63.369	
	857.87	23.7	857.83	13.5372	6	63.368	
	857.83	23.8	857.89	13.5369	6	63.367	
	857.86	23.9	857.82	13.5367	6	63.370	Maximum deviation ± 0.002
Mean.....						63.368

ried to the top of the burette by means of short pieces of rubber tubing. These were partly filled with mercury.

The burette was surrounded by a water jacket, which was attached by means of a rubber stopper passing over the lower end of the burette. A second small tube connected with compressed air passed through the stopper and provided for bubbling air through the water jacket. In order to determine the temperature to at least $\pm 0.02^\circ$ and with care to $\pm 0.01^\circ$, a calibrated thermometer, graduated in rather wide 0.1° divisions was suspended in the water jacket.

A U-tube connected the lowest burette chamber with a manometer tube, closed at its upper end, 120 cm. long, and of capillary bore. In order that capillary attraction should have the same magnitude in the manometer as in the burette, the manometer tube was so chosen that it had a uniform internal diameter closely approximating that of the tubing connecting the burette chambers. Two calibrated thermometers hanging beside the manometer recorded its temperature. At the lowest portion of the U-tube a straight fall tube of about 0.5 cm. internal diameter and about 800 cm. in length was sealed on in order to guard against the possible admission of air. The lower end of this tubing was corrugated in order tightly to fit rubber pressure tubing. Just above the corrugations a small piece of platinum wire was sealed into the glass, so that whenever the mercury was sufficiently raised a closed electrical circuit might be established between the lower wire and each point in the burette.

A levelling bulb was connected with the lower end of the fall tube by means of $\frac{1}{4}$ -inch wall rubber vacuum tubing, which was cemented and wired to glass at both ends. Near the fall tube was placed a tubing clamp through which the rubber tubing passed. The clamp was operated by the foot, and consisted essentially of a hinged lever which could be fastened by means of a ratchet. The levelling bulb was suspended by a small wire rope and controlled in its coarse movements by a reel with a brake; in its finer adjustment by a micrometer screw which moved the reel support.

The manometer was provided with two essential accessories: an electric fan placed over it and directed downwards so as to insure thorough mixing of the air along its sides; and an electric bell so fixed that a rubber hammer could be made to tap the tube rapidly but gently. The former insured a uniform temperature of the mercury column; the latter minimized false readings by causing the column to seek its true level.

When the burette was in its usual position a current passing from a microphone hummer through a high impedance telephone receiver entered the platinum wire sealed into the fall tube, flowed along the mercury column to any desired platinum point in the burette, and left the burette to return to the hummer. By manipulation of a 6-point switch any one of the points could be placed in the circuit. The entire line was grounded and supplied with a paper condenser in order to obviate any hum except when contact was made between mercury and the burette points.

2. *Calibration of the Burette.*—The burette was calibrated with mercury.

In order to calibrate, an ordinary stop-cock was sealed onto the burette, the free end of tubing drawn out to a fine capillary tip and the end of this tip ground off to a flat surface. Above the capillary tip we sealed in a platinum wire in order to provide an electrical connection through the various points. A telephone receiver was placed in series with such a circuit and current supplied from the microphone hummer.

A vacuum pump was connected with one arm of the 3-way stop-cock and pure mercury¹ was slowly drawn into the burette until it was above the level of the upper stop-cock. We then closed the upper stop-cock. After closing the lower stop-cock, the upper stop-cock was turned so as to open to the air. By turning the lower stop-cock carefully, mercury was allowed to flow from it into Pyrex flasks, weighed to 1 mg., until contact with the uppermost platinum point was broken. This was established by

TABLE II.

Volume No.	Chamber.	Volume.	$\frac{V}{760R}$	Capillarity correction.
		cc.		mm.
1	1	1.315	0.21083×10^{-4}	-0.40
2	1-2	3.022	0.48450×10^{-4}	-0.15
3	1-2-3	11.794	0.18909×10^{-3}	-0.20
4	1-2-3-4	22.897	0.36710×10^{-3}	-0.27
5	1-2-3-4-5	49.267	0.78987×10^{-3}	-1.20
6	1-2-3-4-5-6	112.635	0.180582×10^{-2}	-5.20

the fact that very gentle tapping of the burette resulted in loud crackles instead of the musical hum previously heard in the telephone receiver. An empty weighed flask was next substituted under the lower stop-cock and the same process repeated successively with each lower point. The temperature of the burette was noted just after each contact had been broken. Table I indicates the method of calculation and the accuracy of the procedure. This accuracy could, if necessary, be readily increased; for our present purposes a maximum deviation of 0.002 cm. introduced no significant error.

When gas was measured the volume represented when contact was made at any given point was evidently the sum of the volumes of all chambers lying above that point. Since our results are calculated as mols of gas it was convenient to obtain a factor for each volume.

¹ We purified our mercury by bubbling air through it when covered with a layer of dilute nitric acid, passing it through a column of dilute nitric acid, and redistillation in the order mentioned.

TABLE III.

Serial No.	Date.	Volume No.	Absolute temperature of burette.	Observed pressure.	Manometer temperature.	Pressure corrected for temperature.	Capillary correction.	Corrected pressure.	Mols $\times 10^3$	Difference Mols $\times 10^3$	Difference.
	1943			mm.	°C.	mm.		mm.			cc.
252	Apr. 16	5	295.22	715.15	23.4	712.37	-1.20	711.17	0.19028	0.00026	0.056
252a	" 16	6	295.25	317.10	23.1	315.98	-5.20	310.68	0.19002		
253	" 16	6	296.02	266.35	23.5	265.31	-5.20	260.11	0.15868	0.00010	0.022*
253a	" 16	5	296.01	598.70	24.2	596.27	-1.20	595.07	0.15878		
255	" 16	4	296.00	240.95	24.0	239.99	-0.27	239.72	0.029730	0.000012	0.003
255a	" 16	3	296.04	467.35	24.1	465.47	-0.20	465.27	0.029718		
258	Apr. 24	2	296.17	246.55	24.5	245.53	-0.15	245.38	0.004014	0.000024	0.006
258a	" 24	1	296.18	570.10	24.5	567.75	-0.40	567.35	0.004038		
259	" 24	6	296.51	341.65	24.4	340.25	-5.20	335.05	0.20406	0.00001	0.002
259a	" 24	5	296.50	770.40	24.6	767.24	-1.20	766.04	0.20407		
261	" 24	4	296.50	682.70	24.6	679.90	-0.27	679.63	0.084146	0.000070	0.015
261a	" 24	5	296.47	318.60	24.4	317.30	-1.20	316.10	0.084216		
262	" 24	3	296.48	493.45	24.6	491.45	-0.20	491.25	0.031331	0.000124	0.026
262a	" 24	4	296.48	255.35	24.4	254.31	-0.27	254.04	0.031455		
263	" 24	3	296.50	149.90	24.7	149.30	-0.20	149.10	0.009508	0.000022	0.005
263a	" 24	2	296.49	583.00	24.6	580.64	-0.15	580.49	0.009486		

264	"	24	2	296.51	278.85	24.6	277.71	-0.15	277.56	0.004535	0.000008	0.002
264a	"	24	1	296.50	641.90	24.6	639.28	-0.40	638.88	0.004543		
265	"	24	4	296.33	440.70	24.9	438.97	-0.27	438.70	0.054347	0.000062	0.013
265a	"	24	5	296.32	205.65	24.8	204.85	-1.20	203.65	0.054285		
266	"	24	3	296.23	121.80	23.4	121.32	-0.20	121.12	0.007731	0.000043	0.009
266a	"	24	2	296.25	472.10	23.6	470.22	-0.15	470.07	0.007688		

* 0.000045 mols $\times 10^3 = 0.01$ cc.

From the gas law, $PV = NRT$, it follows that $N = \frac{PV}{RT}$. Now V is known for each point, while P and T were determined at each reading. Consequently, $\frac{V}{760 \times 82.07}$ furnished the desired factors. These, together with the volumes, as well as the "capillarity" corrections as derived in a succeeding section, are given in Table II.

3. Burette Manometer Calibration.—In spite of the care exercised to have the capillary between the burette bulbs and that in the manometer of the same internal diameter, capillarity and possibly refraction always caused an error in our manometer readings, and necessitated a correction. This in the case of the smaller volumes was negligible, but reached the very appreciable magnitude of 5 mm. in the case of the largest volume. In order to correct for this error we calibrated the burette and manometer in two ways.

In the first method burette and manometer were thoroughly evacuated. Mercury was raised until contact was made with each point and again lowered until contact was just broken. The latter was evidenced by crackles which appeared in the telephone receiver as the manometer was tapped by the electric bell. The difference between the level of mercury in the burette and in the manometer was then read to 0.05 mm. with a cathetometer. All readings given by the apparatus were made in a similar manner.

In our second method we admitted into the burette a quantity of hydrogen sufficient to give a pressure reading of 100 to 400 mm. on the manometer, using a given volume of the burette. The hydrogen was next compressed sufficiently just to fill the next smaller volume and the pressure redetermined. This process was repeated with each adjacent pair of volumes. In each case the temperature of the burette and manometer was noted.

The observed pressures were next reduced to values in mm. of mercury at 0°C. according to the formula $P_0 = P_1 - AP_1$, where $A = \frac{B - B_1 t}{1 + B_1 t}$ and $B = 0.000182$ = cubical coefficient of expansion of mercury, $B_1 = 0.000138$ = linear coefficient of expansion for the Monel scale of the cathetometer, t = temperature in °C., P_1 = observed pressure and P_0 = pressure in mm. of Hg at 0°C.

A given volume, having no observed capillarity correction, was next chosen as base. Now, since hydrogen is a nearly perfect gas, we could apply the equation $\frac{P_1 V_1}{T_1} = \frac{P_2 V_2}{T_2}$, or $\frac{P_1 V_1 T_2}{V_2 T_1} = P_2$ and obtain a calculated

pressure for the adjoining volume. The difference between the calculated and observed pressures should constitute the capillarity correction for the adjoining volume. By applying such a calculated correction to pressures pertaining to this volume, one could calculate the correction for the next volume; and by repeating the process successively determine a correction for each volume.

Since the values given by the two methods did not agree within 0.5 mm., we used what is essentially a combination of the two to obtain the values for the corrections finally used. In Table III we give the results based on this method. It is to be noted that errors in pressure have of course less effect in the smaller volumes; whereas an error of 0.1 mm. in a pressure of 500 mm. in Volume 6 will cause a difference of 0.015 cc. in volume, under the same conditions in Volume 1 the difference in volume will be about 0.0002 cc.

Our measurements were apparently accurate to about 0.03 cc. in the larger volumes, and to about 0.004 cc. in the smallest. Since the volume of solution introduced into the apparatus varied from 30 to 40 cc. the results, all things considered, should be good to about 0.1 volume per cent when hemoglobin was over 40 per cent saturated with oxygen, and to about 0.015 per cent when saturated to a less degree.

The Equilibrator.

1. *Description.*—The equilibrator consisted essentially of three chambers joined together by glass tubing so as to form a circuit. One chamber, the gas container, could be separated from the other two, which comprised the liquid container, by means of convenient stop-cocks (Nos. 1 and 2).

The gas container was simply a bulb of about 80 cc. capacity which, in addition to the tubes leading to the rest of the device, had another outlet leading upwards and sealed to the rest of the apparatus. This outlet could be closed by a stopcock (No. 3), which was made with a T-shaped bore. One arm of the bore was so drilled that when the stop-cock was closed to the equilibrator the bore was open to the rest of the apparatus. This was done in order to avoid any correction for the bore of this stop-cock which would otherwise have formed a dead space.

The two vessels which, with a connecting tube of 0.6 cm. internal diameter, made up the liquid container, were cylindrical in shape, 3.0 cm. in diameter and 6.0 cm. high. 3 mm. glass rods, fire polished at each end, were packed in each of these chambers so as to nearly fill them, and served to increase the surface of fluid exposed to the gas phase. This arrange-

ment was suggested to us by E. W. Wescott.² In addition to the tubing leading to the gas container each vessel had an additional tube sealed to it. Both were continued to the level of the stop-cocks between the gas and liquid containers. One, which was used in cleaning the apparatus, was otherwise always closed by a small ground stopper carefully sealed at the junction of this tube and the vessel. This tube itself was used for a mercury seal, while the one connecting with the other vessel was used in admitting reduced hemoglobin into the equilibrator. It was about 1.0 cm. in internal diameter and was closed by a 2 mm. bore stop-cock (No. 4) of which it formed one end. Distally, the stop-cock terminated in 4 mm. tubing forming one portion of a ground joint into which another portion, attached to a weight burette, fitted. We used this type of stop-cock to overcome an early difficulty. When smaller tubing led to the liquid chamber, fluid usually completely filled it. And since the solution in the tubing presented only a small surface to the gas, it was doubtful whether equilibrium was ever actually reached in this portion of the fluid. With large tubing leading into the liquid chamber all the hemoglobin solution always ran down the side of the glass into the lower portions of the liquid container.

The entire glass device was mounted in a brass frame and hung on horizontal pivots just above the level of stop-cock 3. A long lever arm, projecting upwards from the frame, was attached to a connecting rod which was moved back and forth by a motor driven eccentric. In this way the apparatus was tilted to and fro about thirty times a minute, thereby causing fluid in one of the cylinders to flow through the large bottom tube into the other. The glass rods in each cylinder greatly increased the surface of the fluid and also served to stir it thoroughly. As a result equilibrium was rapidly attained and yet foaming was avoided, making the addition of any foreign antifoaming substance unnecessary. We believe that this type of device is extremely useful. The entire equilibrator was surrounded by a water bath at a temperature of 37.5°C., heated by means of a 500 watt heating unit and auxiliary carbon lamps. A mercury thermostat and motor driven stirrer provided a temperature uniform to $\pm 0.02^\circ\text{C}$.

2. Calibration.—The equilibrator was calibrated by measuring the hydrogen it contained at atmospheric pressure.

In order to insure uniform results, the equilibrator was first thoroughly evacuated, tested for leakage, and then filled to stop-cock 3 with electrolytic hydrogen under slight pressure. At least half an hour was allowed to elapse in order to assure thermal equilibrium. The entrance stop-cock (No. 4) was then opened and closed several times. Immediately after finally closing stop-cock 4 the barometer was read to 0.05 mm. and both water bath and barometer temperatures were noted. The stop-cocks between the two chambers were next closed. Subsequently the gas in the

² Wescott, E. W., Personal communication.

gas container was removed by a mercury pump of the Töpler type and delivered into the burette, where it was measured. Afterwards the gas in the liquid container was similarly measured. From these measurements the volume of the two chambers could be calculated. By adding a value obtained for the liquid chamber to that of the gas chamber we obtained the volume of the entire equilibrator. Our results are given in Table IV and indicate a maximum deviation of ± 0.03 cc.

3. *Connections and Arrangements for Gas Absorption.*—Stop-cock 3 was sealed to a glass spiral consisting of about twenty-two turns of fine glass tubing, 4 mm. in diameter and of 1 mm. wall. The spiral provided sufficient flexibility to enable us to tilt the equilibrator without breaking the connecting tubing. The fine tubing terminated in a manifold from which tubes led respectively to a Töpler pump of 500 cc. capacity (*B*), gas absorption devices (*D*), and special burette (*C*); a gas burette (*E*) for estimating the amount of gas admitted; and to the Langmuir pump. All these could at will be closed by stop-cocks 6, 7, 8, and 9. When stop-cocks 7 and 8 were closed gas could, with stop-cock 10 open, be collected and delivered

TABLE IV.

Serial No.	Gas container.	Serial No.	Total volume.
	cc.		lcc.
123	82.82	135-137	152.04
124	82.82	136-138	152.03
135	82.80	139-141	152.05
136	82.77	140-142	152.00
139	82.77	143-145	152.02
143	82.83		
Average..	82.80 \pm 0.03 (maximum deviation)		152.03 \pm 0.03 (maximum deviation)

directly into the special burette by means of the Töpler pump. With stop-cock 10 closed and stop-cocks 11 and 12 open it could be dried before delivery. Closing stop-cocks 10, 11, and 12, and opening stop-cocks 13 and 14 enabled us to absorb CO_2 before delivery into the burette for measurement.

The construction of the delivery tube and nozzle should be especially noted. From the Töpler pump a 1 mm. capillary fall tube passed downwards and below the level of bulb *G*, finally terminating in a drawn out nozzle which was sealed into the bulb. Bulb *G* was 3.4 cm. in diameter and was placed in the U-tube connecting burette *C* and the manometer at a point just below the burette. The nozzle was turned slightly upward and situated so as to deliver about one-half way up the bulb and 8 mm. from its wall. In this way we avoided having air bubbles trapped in the U-tube and spattering of mercury in the burette itself. During delivery of gas into burette *C*, the bulb was half filled with mercury and the foot

clamp closed. In this way gradually increasing pressure in the burette caused mercury to flow from the bulb and rise in the manometer. The capacity of the bulb was such that the bulb was never emptied during this process. Consequently gas never was admitted into the manometer tube.

Accessories.

1. *Weight Burette.*—A weight burette fitted with carefully ground stopper at the top and a 3-way stop-cock at the bottom was used to weigh the hemoglobin admitted into the apparatus.

Two arms of the 3-way stop-cock projected downwards. Sealed to one of these was one end of the grinding (16). The lower end was carefully ground off flat. Since hemoglobin in the bore of the stop-cock plug (No. 4), and above it, never was admitted into the equilibrator, it was necessary to determine the volume below the end of the burette tip, including the bore of the stop-cock plug (No. 4).

To accomplish this we determined the volume of the burette tip and the bore in stop-cock plug 15 by calibration with mercury. Subsequently we similarly ascertained the volume from stop-cock plug 15 to stop-cock plug 4 with the grinding always seated in the same way. Now, since observation had shown us that the liquid column broke cleanly at this grinding when the two portions were separated, we could, by difference, determine the volume from stop-cock 4 including the volume of the bore in the plug, to the tip of the burette. This volume correction we found was 0.838 cc. with a maximum deviation of 0.002 cc. and represented the volume of hemoglobin lost by the burette but not delivered into the equilibrator.

In order to determine the volume delivered from the weight burette we obtained the density of the solution to four decimal places by means of an Ostwald-Sprengel type pycnometer; dividing the loss in weight by the density so obtained gave the volume delivered. Deducting the volume correction just mentioned gave the volume of hemoglobin actually delivered inside the equilibrator.

2. *Stop-cocks.*—All stop-cocks except those in connection with parts pertaining to the preparation of gas were carefully selected and of the oblique mercury seal type. When carefully covered with Ramsay grease they held a vacuum for at least 2 to 3 weeks.

3. *Vacuum Pump and Criteria for Vacua.*—A Langmuir type mercury distillation pump backed by a rotary pump provided for evacuation of the entire system down to pressures of 10^{-3} mm. and less.

We always tested the completeness of our evacuation with the spark from a high frequency coil.

This method of testing was suggested to us by Professor Chaffee of the Physics Department of Harvard University. As evacuation proceeded one noted, in a vessel which had previously been filled with air, first the appearance of a red discharge, increasing and then diminishing in intensity. As the red color diminished a greenish fluorescence appeared along the sides of the container. This increased, finally to disappear. Usually, however, we passed only to the stage of marked fluorescence coupled with a grayish blue discharge, the latter undoubtedly due to the presence of mercury vapor. Professor Chaffee assured us that the appearance of greenish fluorescence indicated a gas pressure of the magnitude of 10^{-3} mm.—for our purposes a sufficient vacuum.

Gas Preparation and Storage.

Pure oxygen was prepared by heating potassium permanganate at reduced pressure and stored over mercury.

A sufficient amount of this substance was placed in a Pyrex flask fitted by a ground joint to tubing which was so arranged that the container and adjoining tubing could be evacuated by the Langmuir pump when suitable stop-cocks were opened. The flask was gently heated and evacuation repeated. After thoroughly washing out this part of the system with oxygen in this way, the gas was passed into reservoir *F* where it was stored over mercury. It could readily be delivered into burette *E* in any desired quantity and from this burette into the rest of the apparatus.

Manipulation of the Apparatus.

Hemoglobin, purified as previously described by us (9), and suitably diluted with distilled water, was placed in an Erlenmeyer flask, provided with a carefully fitted ground glass stopper, and thoroughly evacuated. The stopper contained two tubes, one reaching about half way to the bottom of the flask, the other ending flush with the stopper. After connecting the former tube with a rotary vacuum pump, the flask was placed in the water bath at 37.5°C . The latter tube was connected with the underground tip of the weight burette by means of a short piece of rubber pressure tubing. Both burette and flask were exhausted and then refilled with hydrogen. This process was repeated six or seven times. Finally the hydrogen was carefully pumped off and the liquid allowed to flow into the burette by gravity. The weight burette was then disconnected after closing the stop-cock (No. 15). The liquid in the burette was again exhausted for 5 to 10 minutes longer. After carefully drying the inlet tube, the weight burette was weighed to 1 mg.

Grinding (No. 16) was next seated in the same position as in calibration and stop-cock 4 opened. Subsequently the equilibra-

tor was evacuated to the highest vacuum obtainable with our pump. Stop-cocks 1, 2, and 3 were now closed and the solution allowed to run into the liquid portion of the equilibrator by gravity. Here it was equilibrated with a known quantity of oxygen.

A convenient volume of oxygen was next delivered into burette *E* and transferred from it to burette *C* where it was accurately measured. Stop-cocks 5, 6, 9, and 3 were next opened and the larger part of the gas which had just been measured passed into the equilibrator. Since the bore of the plug in 3-way stop-cock 5 could not be emptied after this procedure, a correction for its contents was made in the following manner. The diameter and length of the bore were measured and its volume was calculated. The mercury was now raised in burette *C* until contact was just made with point 1, and the pressure determined. Stop-cock 5 was now closed. From these data one could calculate the quantity of gas trapped in the bore in the plug. In order actually to start the experiment stop-cock 3 was closed and stop-cock 1 opened very gradually and slightly until the liquid was just displaced into the right-hand chamber by the gas pressure. After closing stop-cock 1, stop-cock 2 was similarly opened and the process repeated alternately with each of these cocks until the pressure in gas and liquid chambers was equal. Unless this manipulation was carefully executed liquid passed into the gas chamber and spoiled the experiment.

The motor which actuated the tilting device was now started and run for 20 to 30 minutes, allowing gas and liquid phases to come to equilibrium. During the time consumed by shaking, the residual gas in the connecting tubing was delivered into burette *C* by means of the Töpler pump and measured. Deducting this quantity, together with the amount trapped in the bore of stop-cock plug 5, from the number of mols previously measured in the burette gave the amount of gas actually introduced into the equilibrator. The tubing system and burette were then again connected with the Langmuir pump and thoroughly evacuated.

After again closing stop-cocks 5 and 8 on the manifold, stop-cocks 1 and 2 on the equilibrator, and stop-cock 10, the gas in the gas chamber was passed over phosphorus pentoxide slowly and in small quantities by means of the Töpler pump, alternately opening and closing stop-cocks 12 and 11. The remaining oxygen was delivered and collected in burette *C*, where it was accurately measured. Fifteen manipulations of the Töpler pump were adequate to deliver all but the last traces of gas remaining; in fact, we have calculated that less than 0.0001 cc. would remain after this procedure. We have, consequently, adopted fifteen evacuations as our uniform technique.

When this measurement was completed, stop-cock 3 was again closed, stop-cocks 2 and 3 opened alternately and carefully, after which, as before, equilibration at a new tension was resumed by tilting the equilibrator.

Meanwhile stop-cock 6 was closed, stop-cocks 5 and 8 opened so that burette *C* was again thoroughly evacuated. Again, at the end of a 30 to 45 minute interval, the vacuum pump was disconnected, burette *C* closed, and after shutting stop-cocks 1 and 2, the gas remaining in the gas volume was dried and delivered into the burette where it in turn was measured. In this way the hemoglobin was equilibrated with gradually diminishing tensions of oxygen. In order to again increase the tension a new supply of oxygen could be introduced into the equilibrator and the process, as above described, repeated.

We wish to emphasize that vacua were frequently tested with the high frequency spark during the course of an experiment and that, whenever leakage, however slight, was indicated, the experiment was discontinued. Another point deserving further mention is that the foot clamp was always closed during the delivery of gas into the burette, thereby obviating the danger of delivering air into the manometer.

Calculation of Results.

From the data obtained by the method described above we could calculate our results. Tables V, VI, and VII give typical data.

Table V illustrates how the data appeared as they were obtained. Column 1 is self-explanatory. Columns 2 and 3 refer to the values given in Table II. The temperature of the gas burette (Column 4) was obtained from the thermometer hung in the water jacket and corrected by means of a calibration curve derived by comparison of this thermometer with a U. S. Bureau of Standards calibrated thermometer. The corrected value is given in Column 5. Columns 6 and 7 indicate the readings given with the cathetometer by the upper and lower meniscus of the mercury manometer. The difference column is corrected for the manometer temperature, given in Column 9 in accordance with curves drawn for the formula $P_0 =$

$P_1 - \frac{B - B_1}{1 + Bt} tP_1$. Since the coefficient of expansion of Monel metal is

extremely low we are able to neglect small differences in temperature between the scale and the mercury column. Below the correction due to temperature is given the capillarity correction for the given volume in accordance with Table II. The latter may be taken as constant for the relatively small temperature changes involved. That this leads to no appreciable error is further indicated by our final results. In Column 11 appear the pressures as corrected. Column 12 gives the burette temperature on the absolute scale; Column 13, the factor for the gas volume used as given in Table II. Column 14 gives the result used in our subsequent calculations and denotes the number of mols of gas measured. This quantity was derived from the gas law, and the figures in Column 14 were actually obtained by multiplying the appropriate factor by the corrected pressure (Column 11) and dividing by the absolute temperature (Column 12).

Table VI contains the data from which the amount of hemoglobin admitted into the apparatus was calculated, together with these calculations.

TABLE V.

Experiment 1061. Hb XIX. May 14, 1923.

Serial No.	Volume		Temperature.		Upper meniscus.	Lower meniscus.	Difference.	Mean temperature.	Correction.	Corrected difference.	Absolute temperature. Gas = t + 273.1	$\frac{V}{760H} = D \times 10^3$	$N = D \frac{V}{T} \times 10^3$	Remarks.
	No.	(3)	Observed.	Corrected.										
					(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)	(15)
290	6	112	635.21	05.21	00.712.05	15.40.696.65		22.05	2.57 5.20 7.77	688	88.294.10	1.8059	4.23002	Gas in burette.
291	*	0.092	21.05	21.00	858.00	322.00.536.00		22.20	1.98 0.40 2.38	533	62.294.10	0.00147	0.00267	" " stop-cock.
292	5	49.267	21.26	21.21	852.60	133.25.719.35		23.80	2.85 1.20 4.05	715	30.294.31	0.78987	1.91972 1.92239	" " tubing
293	5	49.267	21.87	21.82	692.30	133.25.559.05		24.10	2.27 1.20 3.47	555	58.294.92	0.78987	1.48798	Gas in gas volume after equilibration I.
294	3	11.794	23.21	23.16	948.80	247.90.700.90		25.78	2.99 0.20 3.19	697	71.296.26	0.18909	0.44532	Gas in gas volume after equilibration II.

* Stop cock.

295	3	11 794 23 84 23 79 500 75 248 00 252 75	25 60 1 08	251 47 296 89	0 18909	0 16016	Gas in gas volume after equilibration III
			0 20 1 28				
296	2	3 022 24 21 24 16 771 00 291 10 479 90	25 80 2 06	477 69 297 26	0 04845	0 07786	Gas in gas volume after equilibration IV
			0 15 2 21				
297	2	3 022 24 48 24 43 588 50 291 10 297 40	26 02 1 30	295 95 297 53	0 04845	0 04819	Gas in gas volume after equilibration V
			0 12 1 42				
298	1	1 315 24 85 24 81 808 35 322 15 486 20	26 20 2 12	483 68 297 91	0 02108	0 03423	Gas in gas volume after equilibration VI
			0 40 2 52				
299	4	22 897 24 90 24 86 753 75 197 50 556 25	26 28 2 43	553 55 297 96	0 36710	0 68200	Gas introduced into burette.
			0 27 2 70				
300	*	0 092 24 95 24 91 391 50 322 25 69 25	25 60 0 25	68 60 298 01	0 00147	0 00033	" in stop-cock.
			0 40 0 65				
301	3	11 794 25 00 24 96 864 50 248 05 616 45	26 11 2 70	613 55 298 06	0 18909	0 38923	" connecting tubing
			0 20 2 90			0 38956	
302	3	11 794 25 16 25 12 515 55 248 10 267 45	25 75 1 14	266 11 298 22	0 18909	0 16873	" gas volume after equilibration VII.
			0 20 1 34				

In Table VII we have brought the data contained in Table V into a more convenient form. Column 3 denotes the number of mols of gas introduced into the burette for measurement, Column 4 the mols left in connecting tubing after introducing gas into the equilibrator; both are obtained from Table V. Their difference, appearing in Column 5, gives the number of mols introduced into the equilibrator, U , the number of mols of oxygen in the gas space, is a measured quantity also derived from Table V and appears in Column 6. Since this amount of gas was removed from the apparatus before a subsequent equilibration, the difference be-

TABLE VI.

Experiment 1061. Hb XIX. May 14, 1923.

Weight of burette + Hb	= 125.3850	Weight of pycnometer full	= 21.4185
Weight of burette empty	= 88.9180	Weight of pycnometer empty	= 16.7693
Weight of Hb	= 36.4670	Weight of Hb	= 4.6492

$$\text{Volume of Hb} = \frac{\text{Weight Hb}}{\delta \text{ Hb}} = \frac{36.4670}{1.0171} = 35.854$$

$$\delta \text{ Hb at } 37.5^\circ = \frac{\text{Weight Hb}}{\text{Pycnometer factor}} = \frac{4.6492}{4.5711} = 1.0171$$

Volume Hb introduced into equilibrator.

= Volume lost from burette - correction for amount above stop-cock 4.

$$= 35.854 - 0.838 = 35.016.$$

tween Columns 5 and 6 gives the amount of gas remaining for the next equilibration. This appears opposite the subsequent equilibration number as amount of gas in apparatus. Where oxygen was admitted into the equilibrator a second time, as before equilibration VII, the amount of gas in the apparatus is the sum of that remaining, as indicated, and the second amount admitted into the apparatus. The latter quantity is derived in the way we have described.

The pressure of oxygen in mm., appearing in Column 7, is calculated by means of the gas law equation $P = N \times \frac{RT}{V}$. Since $T = 37.5^\circ$ and $V = 82.80$, we have evaluated the constant $\frac{RT}{V}$, multiplied by 760 in order to obtain the pressure in mm. of Hg. This constant is equal to 2.3398×10^{-4} , and multiplied by N gives the desired pressure.

Experiment 1061. Hb XIX. May 14, 1923.

TABLE VII.

Serial No.	Equi- libration No.	Mols $\times 10^3$				P_{O_2} mm. $= U \times 10^3$ $\times 2.3398$	Mols $\times 10^3$				
		Introduced into burette.	In tubing.	In apparatus.	In gas space $= U_1$.		In gas phase $U \times W$	In liquid phase.	In liquid phase per cc.	Dissolved per cc.	Combined per cc.
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)
290-293	I	4.23002	1.92239	2.30763	1.48798	348.2	2.10275	0.20488	0.00585	0.00044	0.00541
294	II			0.81965	0.44532	104.2	0.62931	0.19034	0.00544	0.00013	0.00531
295	III			0.37433	0.16016	37.5	0.22633	0.14800	0.00423	0.00005	0.00418
296	IV			0.21417	0.07786	18.22	0.11003	0.10414	0.00297	0.00002	0.00295
297	V			0.13681	0.04819	11.28	0.06810	0.06871	0.00196	0.00001	0.00195
298	VI			0.08862	0.03424	8.01	0.04837	0.04025	0.00115	0.00001	0.00114
299-301		0.68200	0.38956	0.05439							
				0.29243							
				0.34682							
302	VII				0.16873	39.5	0.73844	0.10839	0.00310	0.00005	0.00305

$$C = \text{Vol. Hb} = 35.016 \quad S = \text{Vol. gas phase} = 152.03-C \quad W = \frac{S}{82.80} = 1.4131.$$

$$= 117.01$$

Protein, gm. per cc. = 0.107 Density Hb = 1.017 H₂O per cc. of solution = 0.91.

TABLE VIII.

Experiment No. (1)	Date. (2)	Hb (3)	Serial No. (4)	P_{50} mm. (5)	O_2 combining per cc. mols $\times 10^3$ (6)	100 per cent saturation. (7)	Saturation. (8)	$\frac{Hb}{HbO_2} = R$ (9)	Log R (10)	Log P_{50} (11)
1061	1923 May 14	XIX	290-293	348.2	0.00541	0.00541	100.0	0.0000	$-\infty$	2.5419
			294	104.2	0.00531		98.2	0.0183	-1.7376	2.0179
			295	37.5	0.00418		77.3	0.294	-0.5316	1.5740
			296	18.22	0.00295		54.5	0.835	-0.0783	1.2605
			297	11.28	0.00195		36.0	1.777	+0.2497	1.0523
			298	8.01	0.00114		21.1	3.739	+0.5728	0.9036
			299-302	39.5	0.00305		56.4	0.773	-0.1118	1.5964*
			310-313	219.7	0.00235	0.00264				
1062	May 24	XIX	314	69.4	0.00275					
			315	23.5	0.00255					
			316	10.38	0.00198					
			317	5.99	0.00142					
			318-321	78.8	0.00245					
			330-334	168.3	0.00299	0.00301	99.3	0.00705	-2.1518	2.2261
1063	May 29	XX	335	54.0	0.00266		88.4	0.131	-0.8827	1.7326
			336	20.9	0.00188		62.4	0.602	-0.2204	1.3197
			337	10.02	0.00122		74.5	1.469	+0.1670	1.0009
			338	6.40	0.00059		19.6	4.101	+0.6129	0.8062
			339-342	43.3	0.00184		61.1	0.637	-0.1959	1.6368*

1064	May 31	XX	350-353	239.9	0.00389	0.00391	99.5	0.00503	-2.2984	2.3901
			354	79.4	0.00385		98.5	0.0152	-1.8182	1.8998
			355	29.7	0.00308		78.8	0.269	-0.5702	1.4728
			356	14.06	0.00215		54.9	0.822	-0.0851	1.1480
			357	8.27	0.00136		34.8	1.874	+0.2728	0.9175
			358	5.46	0.00117		29.9	2.344	+0.3700	0.7372
			359	3.65	0.00079		20.2	3.950	+0.5966	0.5623
			360	2.47	0.00053		13.5	6.407	+0.8067	0.3927

* Return.

The data given at the bottom of the table remain constant within the experimental error during the course of an experiment and are used in calculating the values given in subsequent columns. S , equal to the volume of the gas phase, is represented by the difference between 152.03, the total volume of the equilibrator, and C the volume of hemoglobin admitted into it. W is a factor obtained by dividing S , the volume of the gas phase, by 82.80, the volume of the gas container. Since any oxygen removed from the gas space was always saturated with water vapor, we might expect the W to vary with diminution of liquid in the apparatus. The amount of liquid water lost after each equilibration has, however, been calculated and amounts to only 0.0034 cc.—a quantity which is so small in comparison with an error of 0.03 cc. in the volume of the entire equilibrator that it can be neglected even when increased tenfold.

The protein concentration, given in gm. per cc., was obtained by the Kjeldahl method. The density was obtained from Table VI. The difference between these two values gives the number of gm. of water per cc. of solution, or, with sufficient accuracy for our purposes, the number of cc. of water in a cc. of solution.

Column 8 contains values for the product of U , the amount of oxygen in the gas space, and the factor W , which denotes the number of mols in the gas phase. The difference between Column 5 and Column 8 is stated in Column 9 and represents the number of mols of oxygen in the liquid phase. This number divided by C gives the number of mols in the liquid phase per cc. and appears in Column 10.

In order to obtain the number of mols combined we correct for the amount in solution, using a correction suggested by Van Slyke, Wu, and McLean (10), the validity of which is borne out by the experimental work of Findlay and coworkers (11). They suggest that the amount of oxygen dissolved per cc. of solution depends on the amount of water present. We can calculate this value with sufficient accuracy in the following manner. Using the mean of Bohr's and Winkler's values for the solubility of oxygen in water and our value for the number of cubic centimeters of water in each cc. of solution, we obtain a coefficient in the following manner.

$$\frac{\alpha}{760RT} \times \text{vol. H}_2\text{O per cc. solution} = \frac{0.02387}{82.07 \times 273.1 \times 760} \times 0.91 = 1.275 \times 10^{-6}$$

This multiplied by the oxygen tension (Column 7) gives the mols of oxygen in simple solution, given in Column 11. This correction applied to figures appearing in Column 10 gives the number of mols of oxygen combined in each cc. of solution. These values appear in Column 12.

Results.

We have collected data from typical experiments in Table VIII. Using these data we can readily express our results graphically. This has been done in Fig. 2 where the ordinates denote mols of combined oxygen; the abscissæ, oxygen pressures. Curves

have been drawn through each set of points and continued to points corresponding with 300 mm. of oxygen tension, even where the data extended only to pressures substantially lower. Extrapolation in this manner is justified by the data of Barcroft (4) for hemoglobin solutions, confirmed by our present data, as well as the observations of others working with whole blood, which definitely indicate that hemoglobin is nearly saturated with oxygen

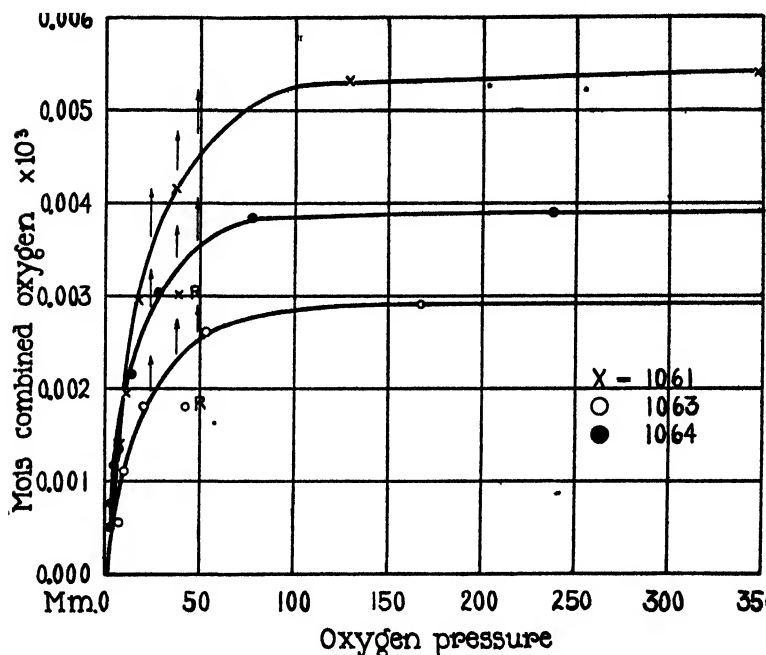


FIG. 2.

at oxygen tensions of 100 mm. Consequently the shape of the dissociation curve over the range of our extrapolation is well established. It is evidently flat. It is to be noted that the curves so drawn are smooth and pass through all but two of the experimental points. These points, marked "R", were obtained when the oxygen tension was increased after successive equilibrations with decreasing tension. Since this deviation from the curves appears for the same points when plotted differ-

ently, as in our other diagrams, we shall discuss their significance later.

The data of Experiment 1062 are unreliable, undoubtedly because of leakage. We have introduced them chiefly to show that a bad experiment is unmistakable, and shall omit them from further calculations and considerations.

In order to have the data from one experiment more directly comparable with those of another and with the data of others we have, in Column 8, expressed our results in terms of percentage saturation at the experimentally determined oxygen tensions.

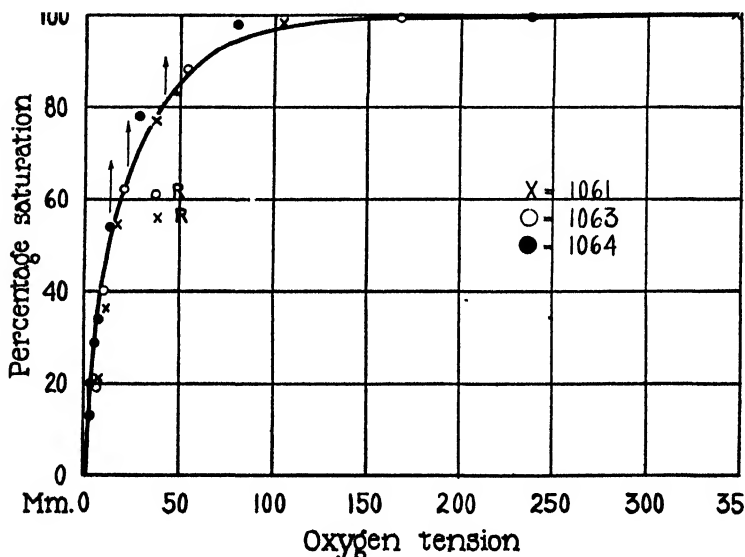


FIG. 3.

For this purpose we have taken the ordinate at 300 mm. of tension in Fig. 2 as representing the amount of oxygen combined when the active hemoglobin present was completely saturated with oxygen. Dividing the value for the amount combined at any other tension by this value (Column 7) gives the percentage saturation, provided the quotient is multiplied by 100. Using values so obtained, we have plotted percentage saturation *B* against oxygen tension in mm. in Fig. 3. A curve has been drawn through the experimental points. It is smooth and closely approximates all but the two points marked "R".

DISCUSSION.

The points obtained by plotting percentage saturation against oxygen tension so nearly fit a single curve that we feel that a definite process is graphically described by them. Careful scrutiny shows, however, that a separate curve, differing but slightly from the one drawn, is needed to fit each set of data. These curves do not cross. Consequently we feel that while their differences may be due in part to the experimental error, it is more probable that they are due to real differences such as changes in the hydrogen ion concentration, and in the effective concentration of hemoglobin in our solutions, which had developed on standing even in the cold room at 2°C. Such differences, the general shape of the curve, and the fact that points marked "R" do not lie upon it, make it clear that the process graphically described in Fig. 3 is not the simple equilibrium between oxygen and hemoglobin.

The observations of Conant (12) suggest that the simple equilibrium is complicated by the simultaneous operation of the equilibrium between met- and oxyhemoglobin. A partial transformation of hemoglobin into the met-form would adequately explain the falling off in oxygen bound, as expressed by the two "Return" points. This fact prevents, for the time being, full utilization of the reversible oxygen-hemoglobin equilibrium in making further measurements. Unfortunately neither our knowledge of the met-oxyhemoglobin equilibrium nor the present data are adequate to attempt an analysis of our curve. Since the amount of active hemoglobin certainly decreased progressively with successive observations, the percentage saturation indicated by our data at a given oxygen tension is too small. It is clear, therefore, that the curve should be displaced in the direction indicated by arrows. One may, however, regard the present curve, particularly at oxygen tensions below 10 mm., as an approximation to the one describing graphically the equilibrium between oxygen and hemoglobin. It possesses slight, if any indications of an S-shape.

We have attempted further to analyze our data by plotting $\log \frac{\text{Hb}}{\text{HbO}_2} = \log R$ as ordinate, against $\log P_{\text{O}_2}$ as abscissa. This

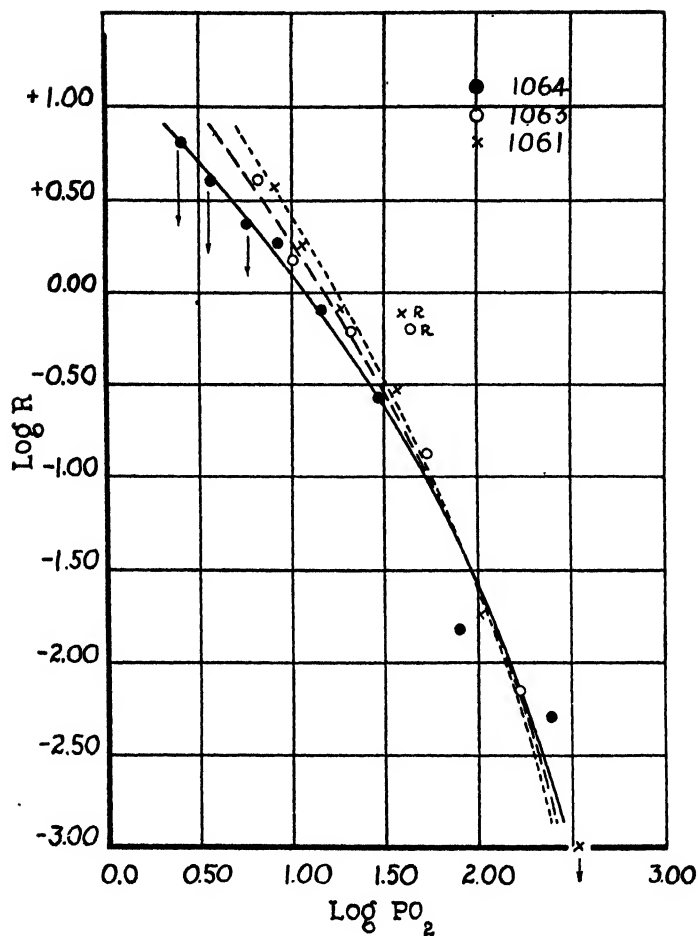


FIG. 4.

method of representation may be used as a simple criterion³ for "Hill's equation", which may be written $K = (O_2)^n \frac{(\text{Hb})}{(\text{HbO}_2)}$. Taking the logarithm of both sides $\log K = n \log O_2 + \log \frac{\text{Hb}}{\text{HbO}_2}$. This is clearly a linear equation, whatever the value for n , and

³ This criterion was suggested to us by L. J. Henderson, and has recently been used by Brown and Hill (13).

may readily be transformed into the form $\log R = n \log O_2$. Clearly if Hill's equation holds, the points belonging to a single set of data must fall in a straight line.

In order to apply this criterion we have added to Table VIII values for the ratio $\frac{Hb}{HbO_2} = R$ (Column 9), and in Columns 10 and 11 values for $\log R$ and $\log P_{O_2}$, respectively. In Fig. 4 we have graphically expressed these values and have drawn smooth curves through each set of points. It is to be noted that the fit of these curves is not so good as with other methods of plotting. We can attribute this; first, to an exaggeration of errors inherent in this method of expression, and secondly, to the fact that values for $\log R$ are uncertain where either Hb or HbO_2 become small. Especially is this the case where $\log R$ approaches $-\infty$ at complete saturation.

Since we are apparently dealing with curved lines, our observations do not seem to satisfy this simple criterion. We cannot, however, on the basis of these data, state that Hill's equation does not hold. In the first place, our points, particularly over the range where $\log R > 0$, should be displaced downwards as indicated by the arrows. This would apparently increase their curvature, but might, conceivably, merely change the slope of the line. Secondly, as became clear to us after making calculations using a different solubility coefficient for oxygen, the choice of solubility coefficient alters not only the slope but the form of our curves. For wherever the oxygen tension is relatively high, the correction to be applied to the oxygen in the solutions becomes greater, or in other words, the value for 100 per cent saturation falls. As P_{O_2} decreases the correction becomes smaller, until at tensions below 20 mm. it has become insignificant. This results in making the ordinary dissociation curve take on a sharper curvature wherever a slightly larger solubility coefficient is used and flattening the curve slightly when a smaller coefficient is applied. Varying corrections entirely change the shape of the $\log R$ - $\log P_{O_2}$ curves.

It must also be remembered that the curves as presented indicate oxygen dissociation at constantly varying hydrogen ion concentration, caused by the change of the weak reduced hemoglobin acid into the stronger oxyhemoglobin acid, demanded by

the theory of Henderson (14). This difficulty can be overcome by heavily buffering the solutions. We were, however, interested in obtaining first the curve for unbuffered solutions, hoping that this would, in connection with curves for buffered solutions, tend to clarify the process studied.

Any explanation of the equilibrium between oxygen and hemoglobin must, then, take into account not only the acid-base equilibrium of reduced and oxyhemoglobin but also that between met-, oxy-, and reduced hemoglobin. These matters are now being investigated in this laboratory.

We wish to thank Miss Madeleine Piggot for technical assistance.

SUMMARY.

1. Previous methods for the study of the equilibrium between oxygen and hemoglobin have been reviewed. They are inadequate in that the order of accuracy is too low either (a) because of apparatus which is incapable of greater precision or because the use and transfer of samples increases the possibility of error, or else that (b) the addition of ferricyanide makes it impossible to take advantage of the reversible equilibrium between oxygen and hemoglobin.

2. A method for studying this equilibrium is described which is, we believe, susceptible of considerable accuracy. This method depends on a study by purely physical means of the gas phase in equilibrium with hemoglobin. The method can theoretically be made continuous in operation, although changes in hemoglobin that have not yet been controlled have so far prevented the application of this principle.

3. Results obtained by the use of this method have been presented. When graphically expressed our data approximate or lie in smooth curves showing slight, if any, indication of an S-shape. The data are not, however, interpretable in terms of a simple oxygen-hemoglobin equilibrium, but involve in addition the equilibria between met-, oxy-, and reduced hemoglobin, as well as the acid-base equilibria of oxy- and reduced hemoglobin.

4. A simple criterion for the validity of Hill's equation, written in the form $K = (O_2)^n \frac{(Hb)}{(HbO_2)}$ is afforded, as shown by Brown

and Hill (13), by plotting $\log O_2$ against $\log \frac{Hb}{HbO_2}$. Experimental points so expressed should, if the equation holds, lie on a straight line no matter what value n assumes. Although our present results do not corroborate Hill's equation, we are unwilling to draw conclusions concerning its validity.

5. It has been shown that slightly larger values for the solubility coefficient of oxygen in hemoglobin solutions tend to make the form of the dissociation curve assume a sharper curvature. Smaller values tend to flatten it. Slight variation in the value of the coefficient changes the form of the $\log R$ - $\log P_{O_2}$ curve.

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ARE GUANIDINES PRESENT IN THE URINES OF PARATHYROIDECTOMIZED DOGS?

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In 1912, Koch (1) reported the isolation of methylguanidine from the urine of parathyroidectomized dogs. In a repetition of this work (2), he again obtained methylguanidine, together with some other bases. A few years later, Paton and his collaborators (3, 4, 5), in a series of papers, pointed out a number of resemblances between the symptoms of intoxication with guanidine and those following parathyroidectomy. These authors definitely concluded that tetany following parathyroidectomy is due to an intoxication with guanidine bases.

This view has won quite general acceptance and has, in fact, almost displaced the theory that a disturbance of inorganic metabolism, particularly of that of calcium, is the immediate exciting cause of tetany. Nevertheless, the experimental evidence for this view is altogether unsatisfactory. It would seem quite obvious that resemblances between guanidine intoxication and tetania parathyreopriva cannot be regarded as proof that the latter is due to the former. Such resemblances can be regarded as significant only if there is some direct evidence of an increased formation or decreased destruction of guanidines after parathyroidectomy. Paton and his associates believed that they had such evidence in the work of Burns and Sharpe (4) who obtained gold compounds of substances believed to be guanidines from the blood and urine of parathyroidectomized dogs in larger amounts than from the blood and urine of normal dogs. They also believed they observed a similarly increased excretion of guanidines in the urines of children with idiopathic tetany. However, the methods employed by Burns and Sharpe were very faulty. For

urines, they used, as Koch had before them, precipitation with mercuric chloride and sodium acetate. It has since been shown by Baumann and Ingvaldsen (6) and by the author (7) that mercuric acetate oxidizes creatine to methylguanidinoglyoxylic acid, which, on evaporation of its solutions, decomposes into methylguanidine and oxalic acid. It is quite evident that the isolation of methylguanidine from materials that may contain creatine must not be attempted by methods that include precipitation with mercuric acetate or mercuric chloride and sodium acetate.

It may be objected that Burns and Sharpe obtained much more guanidines from the urines of parathyroidectomized dogs than they did from the urines of normal dogs. The increased excretion of creatine after parathyroidectomy (8) may have been responsible. Creatinine is promptly and quite completely precipitated by mercuric chloride and sodium acetate. Creatine does not form an insoluble mercuric salt but remains in solution and is quite rapidly oxidized.

Burns and Sharpe also believed that they had isolated increased amounts of guanidines from the blood of parathyroidectomized dogs. They state that no mercuric salts were used in some of the experiments, but they do not indicate in which of the published experiments this was the case. Moreover, their subsequent procedure included the use of platinum and gold salts. Neither creatine nor creatinine appear to reduce platinum salts, but gold solutions are readily reduced by both from the resultant mixtures. Methylguanidine picrate was isolated and identified by its melting point and picric acid content.¹

It is unfortunate that Burns and Sharpe² offer practically no criteria as to the identity of their gold compounds.

"On addition of gold chloride to the platinum-free filtrate we obtained first a crop of deep yellow needles, identified chemically and microscopically as guanidin aurichloride $\text{CH}_5\text{N}_3\text{-HAuCl}_4$. The melting-point was higher than the boiling-point of sulphuric acid. On further concentration and on standing, small quantities of broader deeper yellow needles of methylguanidin aurichloride separated out. In some instances we detected microscopic quantities of dimethyl-guanidin aurichloride which we were unable to free completely from the monomethyl salt. The quantities of

¹ Greenwald, I., Unpublished experiments.

² Burns and Sharpe (4), p. 348.

methyl- and dimethyl-guanidin were in most cases too small to be purified and weighed separately, and in all instances contained traces of reduced gold, rendering an estimation of the gold content useless."

In another paper in the same series, Burns (5) reported the results of some metabolism experiments upon parathyroidectomized dogs and upon dogs receiving guanidine. In only one of the latter was the excretion of guanidine followed. After the intramuscular injection of 0.64 gm. of guanidine hydrochloride into a dog weighing 13 kilos, *without inducing tetany*, there was recovered in the urine of the following 24 hours, 0.048 gm. of guanidine and 0.029 gm. of methylguanidine, or 3.7 mg. of guanidine and 2.2 mg. of methylguanidine per kilo. Without correcting for the amounts normally present in the urines of dogs on a similar diet (0.23 to 0.66 mg. per kilo per day, according to Burns and Sharpe), the amounts excreted account for only 25 per cent of the injected guanidine. If the minute quantities isolated from the blood by Burns and Sharpe are to be regarded as the cause of the tetany of parathyroidectomized dogs, the retention of this relatively huge quantity should certainly have induced tetany. Perhaps it was oxidized or otherwise changed. But the concentration in the blood must have been higher than in the blood of parathyroidectomized dogs for at least part of the 24 hours, for the amount excreted was far greater than was found in the urines of any of the parathyroidectomized dogs studied by Burns and Sharpe. It would seem that if the tetany of parathyroidectomized dogs is to be considered as due to guanidine intoxication because they excreted from 0.3 to 1.5 mg. of guanidines per kilo per day, a dog excreting 5.9 mg. per kilo per day should certainly have developed tetany.

In 1920, Findlay and Sharpe (9) reported the results of the examination of the urine of a woman with idiopathic tetany. In this work, the use of salts that might oxidize creatine was avoided. The urine was treated, successively, with tannic acid, barium hydroxide, sulfuric acid, barium carbonate, and hydrochloric acid, filtering out each precipitate produced, and was then evaporated and treated with absolute alcohol. It was believed that this treatment removed all inorganic salts. From the alcoholic filtrate, a picrate was obtained. Of this, Findlay and Sharpe state:³

³ Findlay and Sharpe (9), p. 436

"The crystals were in the form of needles and plates of a bright yellow colour. They were very regular in size and shape and there was no indication that any base other than *guanidin* was present.

"The nitrogen and melting-point were determined in the crystals from two days in the period of study.

"As the composition of the picrate was nearest that of *dimethylguanidin* the results were calculated as such."

Guanidine picrate crystallizes as needles or as plates but dimethylguanidine, in the author's experience, only as needles. The melting point of their picrate is given by Findlay and Sharpe as 227°, which is sufficiently close to that of dimethylguanidine picrate, 225°. The nitrogen content they report as 28.7 per cent, which is considerably higher than the calculated value, 26.5 per cent. Moreover, half the nitrogen is contributed by the picric acid so that the 2.2 per cent is not only a difference of 2.2 per cent from the calculated value of the total nitrogen, but also a difference of 2.2 per cent from the calculated base nitrogen of 13.3 per cent. This is a very considerable difference and quite sufficient, in the author's opinion, to rule out an identification, based on nitrogen content, of this substance with dimethylguanidine picrate.

In their technique, Findlay and Sharpe made no provision whatever for the removal of creatinine and no effective one for the removal of ammonium salts. According to Lobry de Bruyn (10) absolute alcohol at 17–19° dissolves from 0.62 to 0.67 per cent of ammonium chloride. If the final alcoholic extract obtained by Findlay and Sharpe had a volume as low as 50 cc. the ammonium chloride dissolved therein would form 150 mg. of picrate, an amount sufficient to saturate at least 150 cc. of water.¹ In the course of the work about to be described, the removal of creatinine and ammonia proved to be very troublesome.

In passing, attention may be called to the fact that whereas Koch, working with the urine of parathyroidectomized dogs, found chiefly methylguanidine, Burns and Sharpe obtained chiefly guanidine from both dog and human urine and Findlay and Sharpe, dimethylguanidine.

The theory that guanidine intoxication is the cause of the tetany following parathyroidectomy has received so much credence that it seemed desirable to attempt to develop a method for the isolation of guanidines from urine that would be free from the objection that these substances might have been formed from the

oxidation of creatine or other constituent of the urine and also to develop a method of identification more certain than that of determining the nitrogen content of a picrate in which one-half the nitrogen and at least three-fourths of the molecular weight are contributed by the picric acid or of the gold or nitrogen content of a gold chloride in which four-fifths of the molecular weight are contributed by the gold and chlorine. The method devised by the author in his investigation of the supposed presence of methylguanidine in putrefying meat was found to be inapplicable to urine, for even considerable quantities added to urine could not be recovered. Many other procedures were tried and the following was found to be the most satisfactory.

The urine (1,000 to 1,800 cc.) was first treated with a urease solution prepared according to Folin and Youngburg (11). About 1 gm. of jack bean meal was used for each 300 cc. of urine. The mixture was kept at a temperature between 40 and 50° and acetic acid was added as required to keep the reaction acid to litmus. The decomposition of the urea was apparently complete in 5 or 6 hours but the mixture was usually allowed to stand at room temperature overnight. It was then treated with an excess of basic lead acetate solution and then ammonium hydroxide was added until no further precipitation occurred. After standing about 30 minutes, the volume of the mixture was measured and it was filtered on a Büchner funnel. The volume of the filtrate was measured and it was then treated with H_2S . The precipitate was filtered out and washed. The filtrate and washings were evaporated, under diminished pressure, with the outside bath temperature not over 40°, to a thin syrup. Concentrated hydrochloric acid was then added until the mixture was acid to Congo red, after which 10 cc. in excess and about 500 cc. of H_2O were added. The evaporation, under reduced pressure, was repeated until a heavy crop of crystals appeared. This served to hydrolyze most of the very considerable amounts of acetamide that had previously been formed. The mixture was then treated with about 4 volumes of alcohol, filtered from the precipitated salts, which were washed with alcohol, and the evaporation and precipitation were repeated until there was practically no residue insoluble in absolute alcohol. After evaporating off the alcohol, again under diminished pressure, the residue was taken up in water and treated with a small volume of basic lead acetate solution and enough ammonium hydroxide to precipitate out almost all the lead. The precipitate was filtered out and washed, the filtrate was freed of lead with H_2S and was then evaporated, under diminished pressure, to very small volume. After adding about 2 volumes of alcohol and then 2 drops of a saturated solution of zinc chloride in alcohol for every 100 cc. of urine taken, the mixture was allowed to stand 2 days. The precipitated creatinine zinc chloride was filtered out and washed with a little alcohol. The filtrate and washings were diluted with about 4 volumes of water,

made slightly alkaline with ammonia, and a few drops of lead acetate solution. Under these conditions, the filtrate obtained after treatment with H_2S was perfectly clear and contained no colloidal zinc sulfide, which was otherwise apt to be the case. The filtrate was acidified to Congo red with hydrochloric acid and evaporated, under diminished pressure, to small volume. The treatment with alcohol was repeated until all was soluble in absolute alcohol. The ethyl alcohol was then replaced by isopropyl alcohol and the material insoluble in this solvent also filtered out. Several volumes of water were added and the evaporation was repeated to small volume. The liquid was filtered from a small quantity of oil and then treated with a concentrated solution of sodium picrate and enough sodium hydroxide to make the mixture alkaline and have an excess of about 0.1 per cent. Guanidine, methylguanidine, and dimethylguanidine picrate are precipitated under these conditions, but the small amount of creatinine remaining is not, for it enters into the well known Jaffé reaction. After standing in the

TABLE I.

Melting Point, Picric Acid Content, and Nitrogen Liberated by Distillation with Alkali after Removal of the Picric Acid and the Excess Nitron.

Substance.	Melting point.	Picric acid.	Base nitrogen.
	°C.	per cent	per cent
Guanidine picrate.....	318-323	79.5	14.6
Methylguanidine picrate.....	197	75.8	13.9
$\alpha\alpha$ -Dimethylguanidine picrate.....	225	72.5	13.3
Creatinine picrate.....	212-213	67.0	6.9*
Ammonium picrate.....	276-280	93.2	5.69

* The calculated value of the base nitrogen is 12.3 per cent.

ice box overnight, the crystals, if any were present, were filtered out and recrystallized from a little dilute acetic acid. They were then filtered, dried, weighed, and analyzed. The picric acid content was determined by dissolving in hot water and precipitating with nitron (12), the nitron picrate being filtered on a Gooch crucible and weighed. The cooled filtrate was treated with a few drops of nitric acid, the nitron nitrate was filtered out, the filtrate was neutralized to litmus with sodium hydroxide, and a sufficient excess to give a concentration of about 10 per cent in a volume of about 400 cc. was added. This was distilled into standard acid until the volume had been reduced to about 130 cc. and was then diluted and distilled again. If any considerable quantity of ammonia or amine was obtained in this second distillate, the residue was diluted and distilled again. The third distillate contained only traces of ammonia or amine. Under these conditions, guanidine, methylguanidine, and dimethylguanidine give off all their nitrogen as ammonia, methylamine, and dimethylamine. Creatinine gives off only little more than half of its nitrogen. See Table I.

Using this method, no insoluble picrates were obtained from a number of samples of human urine. If guanidines were added in such amount that the guanidine nitrogen constituted more than 0.5 per cent of the total nitrogen, such excess could be recovered. *This is without correction for the solubility of the picrates.* If this be included, the percentage recovery is increased. The experiments are summarized in Table II.

The urines of six dogs that developed tetany after complete thyroidectomy were examined by this method (Table III). The dogs were fasted or kept on diets of meat and lard in varying propor-

TABLE II.

Recovery of Guanidines Added to Human Urines and Identification of the Picrates.

Substance added.	Volume of urine.	Guanidine nitrogen.		Analysis of picrate.		
		Added.	Recov- ered.*	Melting point.	Picric acid.	Base nitrogen.
	cc.	mg.	mg.	°C.	per cent	per cent
Guanidine.....	1,200	55.0	45.3	316		
	1,080	53.8	26.3	320	79.6	
	1,037	55.4	36.5	322	78.4	14.1
	1,226	52.9	46.3	316	79.7	14.5
Methylguanidine...	1,130	58.1	33.4	196	74.3	12.9
	1,608†	95.6	41.7	198	76.0	13.5
Dimethylguanidine...	1,290	44.5	5.9	223	70.0	13.1
	1,700‡	89.8	45.8	221	72.8	13.0

* Actually weighed as picrate, without correction for solubility.

† Contained 12.2 gm. of nitrogen.

‡ Contained 12.9 gm. of nitrogen.

tions. A few received cod liver oil. Some of them lived only 2 days, others longer. One lived 19 days, showing some tetany on almost every day, before he was killed. The urines of four of these dogs were treated directly, without the addition of any guanidines, and no insoluble picrates were obtained. To six specimens, obtained from five dogs, guanidine, methylguanidine, or dimethylguanidine was added. In each case, the corresponding picrate was obtained. The losses with dog urine were rather greater than with human urine, but, if the amount of base nitrogen exceeded 80 mg., identification was complete. In one case, 0.129 gm. of dimethylguanidine nitrogen was added to a urine

containing 12.9 gm. of nitrogen and 0.011 gm. was isolated and weighed as the picrate. When the guanidine nitrogen constituted only 0.4 per cent of the total nitrogen, not enough of the picrate was obtained to permit of a satisfactory determination of the melting point, but the appearance of the crystals left little doubt as to their identity. The urines of two other dogs (Nos. 15 and

TABLE III.

Recovery of Guanidines from Urines of Parathyroidectomized Dogs, with Identification of the Picrates.

Dog No.	Substance added.	Body weight. kg.	Days.	Nitrogen in urine. gm.	Guanidine added. mg.	Nitrogen recovered* mg.	Analysis of picrate.		
							Melting point. °C.	Picric acid. per cent	Base nitrogen. per cent
1	None.	17	2	6.8	0.0	0.0			
	"	17	4	18.1	0.0	0.0			
	"	17	4	41.8	0.0	0.0			
2	"	18	2	16.2	0.0	0.0			
3	Dimethylguanidine.	11	$\frac{1}{2}$ (12)†	12.9	129	11.0	223	72.4	13.0
	Methylguanidine.	11	$\frac{1}{2}$ (7)†	5.9	110	11.0	201	76.5	13.6
	None.	11	$\frac{1}{2}$ (7)†	5.9	0.0	0.0			
4	Methylguanidine.	15	$\frac{1}{2}$ (8)†	20.5	83.0	Trace.			
	None.	15	$\frac{1}{2}$ (8)†	20.5	0.0	0.0			
5	Methylguanidine.	13	2	4.41	133	36	198	76.5	13.6
6	Guanidine.	9	6	11.3	45.5	Trace.			
15	"	18	$\frac{1}{2}$ (3)†	5.32	109	90	318	79.2	13.6
	None.	18	$\frac{1}{2}$ (3)†	5.32	0.0	0.0			
17	Methylguanidine.	18	$\frac{1}{2}$ (4)†	11.9	282	180	198	75.3	13.1
	None.	18	$\frac{1}{2}$ (4)†	11.9	0.0	0.0			
21	Guanidine.	14	$\frac{1}{2}$ (6)†	16.7	108	73	318	78.1	14.3
	None.	14	$\frac{1}{2}$ (6)†	16.7	0.0	0.0			

* Actually weighed as the picrate, without correction for solubility.

† The urines for the period were combined and divided into two equal portions, to one of which the guanidine was added.

17) that were killed promptly after the appearance of tetany gave similar results.

Because of the failure to recover the added guanidines in larger proportions, the experiments are not as conclusive as might be desired. They do not prove the absence of guanidines from the

urines of parathyroidectomized dogs, but it is extremely questionable if any method of examination could do that. It is believed, however, that they do prove that guanidine nitrogen does not constitute more than 0.5 per cent of the total nitrogen of such urines, even when this nitrogen is entirely endogenous (Dogs 4, 15, and 17) or as little as 0.17 gm. per kilo per day (Dog 5).

Together with the reasons that have been advanced for questioning the validity of the conclusions of Burns, Burns and Sharpe, and Findlay and Sharpe, these experiments seem to offer good reason for refusing to accept, without further evidence, the idea that tetany is due to guanidine intoxication.

There are two, and only two, well authenticated metabolic changes after parathyroidectomy. One is the lowered calcium content of the serum or plasma and the other is the diminished excretion of phosphorus in the urine. These must be regarded as being intimately connected with the sequence of symptoms observed. No theory of the causation of tetany can be considered adequate if it fails to take both of these into consideration.

I am indebted to Mr. Joseph Gross for his assistance with the experimental work.

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THE NUTRITIVE VALUE OF LACTALBUMIN.*

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Several years ago, in experiments dealing with the relative nutritive values of proteins from different sources, we¹ arrived at the conclusion that "lactalbumin" is protein of good quality, judged by its capacity to satisfy the needs of growing rats. As ordinarily employed "lactalbumin" is a mixture of the coagulable proteins, including both the albumin and globulin, that remain in milk after removal of the fats and casein by precipitation with acid. Our earliest studies were made with mixtures consisting of lactalbumin 18, starch 28, butter fat 18, lard 8, and "protein-free milk" 28 per cent. The latter was used as a source of inorganic salts and what is now termed vitamin B. The animals grew well on such a mixture, even when the lactalbumin content was as low as 9 per cent.

In various publications McCollum has denied the validity of our earlier conclusions. His views are summarized by the statements² that "lactalbumin as the sole source of protein cannot support any growth whatever" and that "lactalbumin is actually an incomplete protein and is incapable of inducing any growth whatever unless its deficiencies are made good by some other

* The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, D. C.

¹ Osborne, T. B., and Mendel, L. B., *Z. physiol. Chem.*, 1912, lxxx, 307. Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1915, xx, 351; 1916, xxvi, 1.

² McCollum, E. V., *The newer knowledge of nutrition*, New York, 2nd edition, 1922, 70.

source of amino-acids.”³ According to McCollum’s view, the successful growths which we observed in our earliest feeding experiments with lactalbumin were due to the supplementary effect of the nitrogenous components remaining in the protein-free milk used. In further support of this assumption he refers to later experiments reported by us⁴ wherein less success was attained with lactalbumin when dried brewery yeast was incorporated with the food as a source of vitamine B instead of the protein-free milk. The implication is naturally made that the protein-free milk furnishes a sufficiency of one or more essential amino acids in which lactalbumin is relatively or absolutely deficient, whereas yeast though it contains protein does not yield these necessary supplements in the same relative abundance. McCollum, Simmonds, and Parsons⁵ reported, further:

“We have been unable to obtain any growth in young rats with diets containing 18 per cent of lactalbumin as the sole source of protein, when the remainder of the food mixture was so constituted that good growth could be secured when the protein (18 per cent) of the diet was casein.”

The details of their experiments are not given, so that the source and quantities of the vitamine B and other essentials used cannot be considered critically. Experience has taught us that the adequacy of the vitamine intake must always be carefully scrutinized in the interpretation of experimental failures of nutrition.

Sure,⁶ who also reached the conclusion that lactalbumin “is biologically an incomplete protein,” believed it to be deficient in cystine and tyrosine. In his experiments an alcoholic extract of ether-extracted wheat embryo supplied the vitamine B. How much of the latter each animal secured per day is not apparent from the published data. Several of the charts indicate that the animals grew fairly well for some time before the alleged deficiency clearly manifested itself by slowing or cessation of growth. One rat, Chart II, No. 17, for example, grew well to a size of 200 gm. without any amino acid supplement. The author endeavored to

³ McCollum, ² p. 102.

⁴ Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1917, xxxi, 149.

⁵ McCollum, E. V., Simmonds, N., and Parsons, H. T., *J. Biol. Chem.* 1919, xxxvii, 289.

⁶ Sure, B., *J. Biol. Chem.*, 1920, xliii, 457.

explain this successful growth on a lactalbumin diet by remarking that the ability "no doubt was resident in either a larger consumption of the ration, or else in a greater efficiency in conserving available sulfur complexes in the body."⁷ One might remark with equal or greater probability that the animals which failed to grow so well or ceased to show good gains at a comparatively early period did so because of an increasing deprivation of vitamine B. We⁸ have shown that a daily dosage of vitamine B that just suffices for an 80 gm. rat will not prove sufficient to promote normal nutritive conditions in a 200 gm. rat. When the vitamine is incorporated in the ration the fact that rats eat proportionately less as they grow large will tend to result in a proportionately smaller daily intake of the included vitamine; so that when the content thereof is near the lower limit of requirement at the outset it may ultimately prove insufficient.

Emmett and Luros⁹ have described good growth in rats on diets in which vitamine B was supplied by extract of wheat germ (which Sure and perhaps McCollum have employed) as well as by extracts of yeast. They have come to the conclusion: "Lactalbumin is a complete protein in the sense that it does not lack any of the nitrogenous cleavage products essential for growth," although their results lead them to assume that lactalbumin is peculiar in its nutritive properties. The results are not easy of interpretation.

In our own failures with lactalbumin-yeast foods⁴ we were still following the plan of incorporating the yeast in the food mixture. A review of the food intakes of the animals involved in our report of the experiments (see Rats 3868, 3258, 3242, Chart II) shows that the consumption of the mixture during the periods of unsatisfactory growth was actually below the average intake of the same types of food by rats of the same size during periods of satisfactory growth. Consequently the failure to grow may have been due to inadequate intake of vitamine B as well as insufficiency of the hypothetical supplementary nitrogenous components of the yeast. However, calculations of the actual average daily intakes of yeast during the periods of slow growth have given figures which, in view of our quantitative studies⁵ of the requirement of this source

⁷ Sure,⁶ p. 459.

⁸ Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1922, liv, 739.

⁹ Emmett, A. D., and Luros, G. O., *J. Biol. Chem.*, 1919, xxxviii, 147.

of vitamine B, should be sufficient though near the minimum. We are, therefore, at a loss to explain the failure of the rats to grow better on lactalbumin, edestin, and cotton seed globulin in the experiments under criticism.⁴

In order to secure further evidence regarding the possible inadequacies of lactalbumin as a food protein in the light of the criticisms and evidences thus far recorded, we have undertaken a few new experiments planned to exclude as far as possible any extraneous sources of protein that might act to supplement the milk protein used. At the present time no preparation containing vitamine B is available that does not include some nitrogenous ingredients. In order to exclude the latter to the minimum necessitated by the present limitations in the isolation of vitamine B, yet insure a sufficiency of this food factor, we have employed the yeast fraction described by Osborne and Wakeman.¹⁰ The absence of protein in this product is indicated by its failure to give the delicate biuret reaction. The daily dose (40 mg. used unless otherwise indicated) has been demonstrated to supply the needs of a small growing rat until it reaches a considerable size.¹¹

The lactalbumin was prepared as follows:

The filtrate from casein which had been precipitated with HCl at its isoelectric point was filtered *clear* through paper pulp and heated to boiling. The coagulum was filtered out on cheese-cloth and washed three times by suspending in boiling water and filtering. After pressing in a hydraulic press the coagulated lactalbumin was digested with 95 per cent alcohol, ground therewith to a coarse powder, and boiled out three successive times with 81 per cent alcohol, then pressed and dried in a current of warm air.

The food mixtures had the following composition.

	<i>per cent</i>	<i>per cent</i>
Lactalbumin	9	20
Salt mixture*	4	4
Starch	63	52
Lard	12	12
Butter fat	9	9
Cod liver oil	3	3

* Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxvii, 572.

¹⁰ Osborne, T. B., and Wakeman, A. J., *J. Biol. Chem.*, 1919, xl, 383.

¹¹ A detailed report of the comparative requirements of rats of different sizes and ages for this vitamine when it is the sole source of vitamine B in an otherwise adequate diet will soon be published.

The vitamine preparation was fed, incorporated with starch, in the form of tablets apart from the rest of the food mixture.

The results of a number of feeding trials with foods containing 20 or 9 per cent, respectively, of lactalbumin are shown in Chart 1. The fact that rapid gains, as indicated therein, were secured even with as little as 9 per cent of lactalbumin in a food mixture comparatively rich in fat (so that the total intake of protein in the food of high caloric value would inevitably be comparatively small) is strong presumptive evidence of the high value of lactalbumin as a protein nutrient without any effective supplement in such quanti-

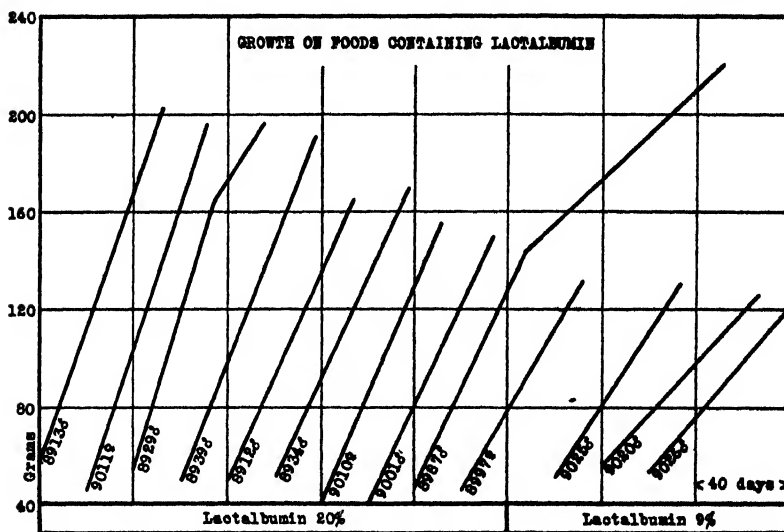


CHART 1.

ties as might be assumed to be requisite if the protein were as defective as one has been led to suppose. Considering the possible criticism that both the so called protein-free milk and the actually protein-free yeast fraction serve to permit such gains on the lactalbumin food, one must assume either that both of these unlike sources of vitamine B furnish precisely the same lacking ingredients or else that lactalbumin is in fact a protein of comparatively good nutrient quality under dietary conditions in which the vitamine supply is adequate. The latter seems to us to be the more probable conclusion, particularly as 40 mg. of the yeast

fraction furnished at most 3 mg. of nitrogen per day. On an average intake of 7 gm. of the 20 per cent lactalbumin food per day the yeast fraction nitrogen would represent something approximating 1 per cent of the nitrogenous intake. Inasmuch as not more than an extremely small fraction of this represents any single amino acid, the improbability that the vitamine preparation made good any amino acid deficiency of the lactalbumin is accentuated. At any rate these experiments represent, we believe, as near an approach to the experimental test of the possible nutritive value of lactalbumin as can be formulated with the present limitations of the feeding technique.

The use of "protein-free milk" in experiments with proteins has been severely criticized on the ground that all of its nitrogen may be equivalent to protein nitrogen in meeting the nutritional needs of the test animals. Thus McCollum¹² has stated with respect to our earlier experiments that involved the use of protein-free milk:

"The importance of their deductions is so great and the defects in their method so difficult for the inexperienced to appreciate, that a somewhat full discussion is warranted of the manner in which 'protein-free milk' vitiated a considerable part of a research of a most comprehensive type."

Our experiments on the rôle of protein in growth have given evidence that notable amounts of certain essential amino acids must be available before their effect is revealed by growth. Thus it is now known that gliadin yields about 1 per cent of lysine; yet when young rats are fed on an otherwise adequate diet containing 18 per cent of this protein, and vitamine B is supplied daily by tablets containing 30 mg. of a yeast fraction (a dose demonstrated to be ample for normal growth in small rats and containing only about 2 mg. of nitrogen), they do not grow at all. The lysine furnished by the gliadin is no more than is needed for maintenance. Furthermore, when diets containing 28 per cent of "protein-free milk," 18 per cent of zein, and 0.5 per cent of tryptophane are fed no growth whatever ensues until lysine is added, thus proving that whatever quantity of lysine may be yielded by the "protein-free milk" it is not more than enough for maintenance.

¹² McCollum,² p. 67.

When young rats are fed on diets containing 28 per cent of "protein-free milk," 13.5 per cent of zein, and 4.5 per cent of casein, which yields at least 1.5 per cent of tryptophane, almost no growth is made until more tryptophane is added. They then grow rapidly, thus demonstrating that the tryptophane furnished by the casein was very little more than is necessary for maintenance.

The experiments just cited clearly show that the "protein-free milk," even when fed in such large proportion of the food, does not furnish enough of either tryptophane or lysine to affect the experiments by which a qualitative deficiency of the protein in these two essential amino acids has been regarded as demonstrated. This is as we should expect, because our careful analyses of "protein-free milk" showed it to contain about 2.2 per cent of protein which on digestion must yield many different kinds of amino acids. Furthermore, it is known that a considerable part of the remaining nitrogen belongs to non-protein substances other than amino acids. Obviously, therefore, no great part of the nitrogen of the "protein-free milk" belongs to any one of the essential amino acids; and, consequently, it cannot supplement to an important degree any amino acid deficiency of the proteins of the diet unless the latter are fed in very small proportion. These facts are important, for they show that so far as growth experiments are concerned the nitrogen of the vitamine component does not introduce as serious errors as has often been alleged. Nevertheless, in experiments designed to determine the nutritive value of proteins, nitrogen in unknown forms should be excluded as far as possible; and every effort to improve the conditions of feeding tests with proteins in this respect should be promoted.

CREATININE AND CREATINE IN MUSCLE EXTRACTS.

V. A COMPARISON OF THE RATE OF CREATININE FORMATION FROM CREATINE IN EXTRACTS OF BRAIN AND MUSCLE TISSUE.

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Myers and Fine (1) and the writer (2, 3) have shown that creatinine is formed from creatine in muscle tissue, or muscle tissue extracts on incubation or autolysis. This is presumptive evidence that such a change is possible in the living organism. It has been the part of Benedict and Osterberg (4) to present the clinching evidence that the living body can convert creatine into creatinine. This finding would seem to confirm the validity of the interpretations of Myers and Fine and the writer; hence, the validity of their method of approach to the problem of creatine metabolism.

Nevertheless, before proceeding with further experimentation along this line it seemed essential that a determination of the relative rate of creatinine formation from creatine in muscle extract and in solutions of pure creatine in the Tyrode's solution used for making the muscle extract, be made. This has been done

The velocity constant k (from the equation for a monomolecular reaction $k = \frac{l}{t} \log \frac{a}{a-x}$) for the transformation of pure creatine to creatinine in Tyrode's solution, buffered to neutrality with phosphate mixture, under toluene, during 24 hours at 38° was found to be 0.00058. That for muscle extract similarly buffered and simultaneously incubated was 0.00119. The detailed data are given in Table I. The use of the above formula in this reaction is justified by results previously reported (3) and the later study of Edgar and Wakefield (5).

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The fact that the rate of creatine transformation in muscle extract is twice as great as that in the extracting medium under identical conditions establishes my previous conclusion "that muscle tissue provides a milieu particularly favorable for this change" on a solid foundation.

Is this biocatalysis peculiar to muscle?

The question is of importance since there are two views with regard to the origin of urinary creatinine.

Folin and Denis (6) hold that creatinine formation is an index of total normal tissue metabolism. Shaffer (7) contends that such is not the case and that the process is essentially confined to muscle tissue. In this opinion Myers and Fine (8) concur. Benedict and Osterberg favor the view of Folin and point out that Shaffer's findings "since muscular tissue represents so large a proportion of the living tissue in the body, . . . would probably obtain whether creatinine production were confined to the muscles or not."

In order to throw some light into the matter I have simultaneously incubated extracts of brain and muscle tissue prepared as described in an earlier paper (2).

The brain was chosen because of its relatively high creatine content, as shown by Beker (9) and Janney and Blatherwick (10).

The methods of analysis and the mode of procedure have been given (3, 11). The animals were all old discards of both sexes. Fifteen rats were needed for each test. The muscle used in the experiments came from the same animals from which the brains were obtained. In this way the best possible controls were used.

The results of the analyses and the velocity constants are given in Table I. The values of k represent the rate of transformation of creatine into creatinine.

The data show definitely that the rate of creatine transformation to creatinine is practically the same in brain tissue as in muscle tissue, under the conditions of experimentation, and that the brain, as the muscle, provides a milieu particularly favorable for this change.

The finding is strong support for Folin's idea that creatinine formation is a property of total normal tissue metabolism, and is

against Shaffer's conception that the process is essentially confined to muscle tissue. It is incomplete support since it deals with only one other type of tissue.

The only other argument that can be raised against the interpretation is that results of experiments of this type should not be considered as indices of processes taking place in the intact organism. I have attempted in the earlier part of this paper to show that in the problem in question, *in vitro* findings have been confirmed by *in vivo* experiments. Moreover, if we are to throw out

TABLE I.

Preformed and Total Creatinine in Fresh and Incubated Extracts of Brain and Muscle Tissue and the Rate of Transformation (k) of Creatine to Creatinine.

Creatinine.	Brain.					Muscle.				
	Fresh.		Incubated.			Fresh.		Incubated.		
	Preformed.	Total.	Preformed.	Total.	k	Preformed.	Total.	Preformed.	Total.	k
	mg.	mg.	mg.	mg.		mg.	mg.	mg.	mg.	
I	0.030	1.80	0.093	1.82	0.00143	0.055	4.24	0.153	4.23	0.00094
II	0.024	1.61	0.068	1.58	0.00115	0.037	4.68	0.166	4.65	0.00119
III	0.023	1.76	0.059	1.74	0.00083	0.032	4.83	0.142	4.77	0.00099
IV	0.017	1.50	0.043	1.51	0.00095	0.029	4.92	0.143	4.86	0.00091
V	0.022	1.77	0.076	1.79	0.00148	0.029	5.55	0.172	5.53	0.00119
Average	0.023	1.69	0.068	1.69	0.00117	0.036	4.84	0.155	4.81	0.00104

Pure creatine in buffered Tyrode's solution.

	0.010	3.78	0.058	3.76	0.00058	0.010	3.78	0.057	3.78	0.00058
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from our conceptions of biological processes all the ideas derived from experiments in which a study was made of changes taking place in incubated tissues, or tissue extracts, many of the hypotheses which are so fondly cherished would fall to pieces. It is acknowledged that no single method of approach to the solution of a problem is all that is necessary or sufficient to solve the question in multiorganic organisms. For the present, however, I can see no other way than that employed here which will advance our information with regard to the point under discussion.

350 Creatinine-Creatine in Muscle Extracts. V.

There are several other matters of interest brought out by this study that will be briefly noted.

According to Koch and Koch (12) the brain of the albino rat of about the age of the animals used in this study contains about 10 per cent of protein. According to Mathews (13) the protein content of muscle tissue (species not given) is about 20 per cent. This being so and assuming with Folin that the creatine is a part of the living vertebrate protoplasm (protein), the creatine content of unit protein of brain is seen to approximate that of muscle. The respective values are 2.0 and 2.8. The difference in absolute values is largely due to the high lipoid content of the brain. It would be a waste of time to speculate on this relation since the data are only approximations.

It is evident that in the brain as in the muscle there are no enzymes destructive of creatine or creatinine, since the total creatinine is the same in the incubated samples as in the fresh material. Nor is there any evidence of creatine formation.

It is possible that the rate of creatinine formation tends to be greater in the brain than in muscle. If this is so, it may be that it is an age phenomenon, since the mean rate in a series of eleven tests with muscle from younger rats was 0.00168 with a probable error of but 0.00004. It is my intention to study the rate of this change on age.

This is the first demonstration of the presence of creatinine in brain tissue. It is, however, possible that the small amount found is but the product of the creatine transformation occurring during the preparation of the extract.

SUMMARY AND CONCLUSION.

Data are presented which show that the rate of creatine transformation to creatinine is practically the same in extracts of brain tissue as in extracts of muscle tissue, when the two extracts are obtained from tissues from the same animals, using Tyrode's solution as the extraction agent and phosphate mixture as buffer, during simultaneous aseptic incubation at 37-38°. This rate is twice as great as that of creatine in buffered Tyrode's solution.

It is, therefore, concluded that creatinine formation is not a process essentially confined to muscle tissue, but is a property of brain tissue as well.

The findings are experimental support for the hypothesis of Folin that creatinine formation is a property of the total normal tissue metabolism.

In addition, it is shown that in the brain as in the muscle there are no enzymes which produce creatine or which destroy creatinine or creatine.

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THE OXYGEN AND CARBON DIOXIDE DISSOCIATION CURVES OF HUMAN BLOOD.*

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For many years studies of the combination and dissociation of oxygen and hemoglobin in blood have been based on the dissociation curve of blood as presented by Barcroft (1). This curve is an S-shaped curve, described by Hill's (2) equation $\frac{y}{100} = \frac{Kx^n}{1 + x^n}$ in which y is the percentage saturation of blood with oxygen, x equals the oxygen pressure, and K and n are constants. Assuming that molecules of hemoglobin fall into aggregates in the presence of salts, Barcroft has interpreted n in the formula to mean the number of molecules in each aggregate, and K is regarded as the equilibrium constant. Owing to the conception that there are varying degrees of molecular aggregation of hemoglobin the value of n is taken as the average value of the aggregates present, 2.5 being the value best fitting the experimental curves thus far determined. Recent studies indicate that 2.2 is a more exact estimate for n . The variations in the dissociation curves of blood of different individuals is considered to be due to change in the value of K only.

An attempt to verify Barcroft's curve with analyses of blood done with the Van Slyke apparatus failed to confirm previous work and led to the production of dissociation curves on the blood of two of the authors (A. V. B. and G. S. A.) that are not capable of definition by Hill's equation. The variations found are greater

* This is study No. 37 of a series of studies on the physiology and pathology of blood from the Harvard Medical School and allied Hospitals, a part of the expense of which has been defrayed by the Proctor Fund for the study of chronic disease.

than experimental errors would appear to account for, and aside from theoretical considerations concerning the relations between hemoglobin and oxygen, many practical points make it advisable to publish oxygen dissociation curves as determined with the apparatus of Van Slyke. In this country the Barcroft-Haldane blood gas methods are little used. If the discrepancy of our results with those of previous workers is a matter concerned with the type of apparatus used, it seems essential that data obtained with the Van Slyke apparatus should be referred when desirable to dissociation curves obtained with this method. The most accurate method at present of obtaining the pressure of oxygen in arterial and venous blood, for example, is based upon the oxyhemoglobin curve, and the value obtained, as shown by the work here presented, varies greatly, apparently depending on the blood gas methods used.

This paper, therefore, presents two well defined oxygen dissociation curves for human blood at a CO₂ tension of 40 mm. of Hg as determined with the apparatus of Van Slyke. Similar curves on the blood of one of us (A. V. B.), at the following tensions of CO₂, 3, 20, and 80 mm., are given, and Barcroft's curve is reproduced for comparison.

Because of the general interest in the relationship of oxygen and carbon dioxide in blood, the average CO₂ dissociation curves on oxygenated and reduced blood of one of us (A. V. B.), as well as the corresponding true plasma curves, are also given. The complete set of curves for both oxygen and CO₂ for the same human blood may be of interest to other workers in this field.

Methods.

Oxyhemoglobin Dissociation Curves.

1. A set of tonometers of approximately 250 cc. capacity was prepared with known gas mixtures, CO₂ about 40 mm. of pressure, O₂ from 2 to 100 mm.

2. After a rest period of 15 minutes about 25 cc. of blood were drawn, without stasis, from the arm vein of the subject, run at once into a tube containing a minimum amount of carbonate-free potassium oxalate to prevent clotting, and stirred gently with a glass rod. A sample of 2 cc. was taken at once for the first tonometer; the rest of the blood was put on ice.

3. The tonometer was rotated for 20 minutes in a water bath at 37.5°C. An equilibration experiment was carried out to determine the optimum time required for equilibrium to be reached between the blood and the atmosphere in the tonometer. The time found was from 17 to 20 minutes. After equilibration 1 cc. of blood was removed from the tonometer in an Ostwald pipette, calibrated to deliver between marks, and run directly under water into the Van Slyke pipette, the burette of which was calibrated to read to 0.01 cc. The determination of oxygen in the sample was carried out according to the technique described by Van Slyke and Stadie (3). The oxygen was absorbed by pyrogallol after the CO₂ had been removed with 0.2 N NaOH. Blank determinations were carried out to find how much gas was present in the reagents. The tiny bubble formed could not be estimated exactly but a maximum error of 0.005 cc. would occur if no correction were made for this bubble. The solubility corrections for oxygen were taken from Van Slyke and Stadie's table, calculated from Bohr's solubility coefficient.

4. The partial pressures of oxygen and carbon dioxide in the tonometer were determined in the usual way in the Haldane gas apparatus. The equilibration of the blood in the tonometer was carried out with a small positive pressure. Since some uncertainty has been felt as to the method of calculating partial pressures in the water bath from experimental data at room temperature, it seems advisable to state the formula used in the calculations.

$$x = p(B - w + pp) \frac{273 + 37.5}{273 + t} \frac{V + 7 - 2 + 1}{V - 2}$$

x = partial pressure of CO₂ or O₂.

p = percentage of gas.

B = barometric pressure.

w = vapor pressure at temperature t .

pp = positive pressure measured in burette of Haldane apparatus.

t = room temperature.

V = volume of tonometer.

7 = " " gas taken into Haldane apparatus."

2 = " " blood present in the tonometer.

1 = " " removed for analysis.

This formula is based on Dalton's law; the pressure of water vapor at 37.5° does not enter into the calculation because the system is an heterogeneous equilibrium.

5. The partial pressure of carbon dioxide desired in the tonometer was 40 mm. of Hg. An inspection of our data will show that while most of the equilibrations were carried out at this tension or within 2 or 3 mm. of it, numerous points were done at somewhat wider ranges of CO₂ tension. In order to correct all points to a CO₂ tension of 40 mm. of Hg use was made of a correction curve obtained by application of a new formula derived from Hill's equation, and a formula published by one of us (G. S. A.) in 1921 (18),

$\frac{1}{K} = au + b$. In the new formula

$$x_{40} = x_u \frac{(40a + b)}{(au - b)^{\frac{1}{n}}}$$

x_{40} = tension of oxygen at 40 mm. of CO₂.

x_u = observed tension at u mm. of CO₂.

a and b = empirical constants.

The equation $\frac{1}{K} = au + b$ has been shown by Hill to be inaccurate when a wide range of CO₂ tensions is considered, but as an empirical formula it is accurate over the range we require.

Carbon Dioxide Dissociation Curves.

For the determination of carbon dioxide in whole blood and plasma the technique used was that described by Austin and his associates (4) with certain minor modifications. Simple gas burettes were used for approximate estimation of the tensions of oxygen and carbon dioxide required in each tonometer and the exact tensions were determined by means of the Haldane apparatus after the blood was removed from the tonometer. The analyses for carbon dioxide were made with the constant volume apparatus of Van Slyke, equipped with a side trap in which the blood reagent mixture was trapped during absorption of carbon dioxide by sodium hydroxide. This modification of the original apparatus enabled us to get more accurate readings than appeared possible when it was used without the side trap.

TABLE I.

Date.	CO ₂	O ₂ (corrected to 40 mm. CO ₂ pressure).	Saturation with O ₂ .	O ₂ capacity (combined).
<i>1928</i>	<i>mm.</i>	<i>mm.</i>	<i>per cent</i>	<i>vol. per cent</i>
June 19	46.0	9.5	13.5	21.7
	45.4	23.7	51.5	
June 20	41.7	10.8	23.7	22.4
	44.4	22.9	49.5	
June 21	47.4	43.2	77.8	
	39.7	53.6	93.8	22.2
June 23	38.2	64.2	91.7	
	43.9	9.6	16.1	22.8
June 26	41.7	63.5	94.2	
	41.8	57.3	91.5	
June 27	41.0	63.0	91.5	22.6
	43.0	96.6	97.6	
June 30	41.6	110.0	97.5	
	45.7	110.5	95.0	21.6
July 1	41.6	13.7	22.8	
	42.2	19.3	36.1	23.0
July 5	41.0	13.9	27.5	
	42.9	110.0	98.6	
July 14	39.2	27.0	51.5	
	41.6	38.5	70.1	23.0
July 17	40.6	27.8	54.5	
	40.4	36.5	71.0	
July 26	41.6	4.2	4.6	21.9
	42.4	4.9	5.0	
July 30	43.0	49.0	84.5	22.2
	38.4	57.0	90.0	
August 3	40.7	64.6	91.4	
	43.4	71.0	95.0	
August 10	35.5	7.6	8.7	
	40.3	6.0	6.6	
August 17	33.8	29.7	66.6	
	45.2	5.2	5.9	21.3
August 24	38.8	5.0	5.9	
	40.0	6.3	6.8	
August 31	42.4	5.0	5.3	22.5
	46.0	27.1	56.6	
September 7	37.5	45.6	83.2	
	42.4	2.5	2.4	23.3
September 14	41.6	2.8	1.7	

358 O₂ and CO₂ Dissociation Curves of BloodTABLE I—*Concluded.*

Date.	CO ₂	O ₂ (corrected to 40 mm. CO ₂ pressure).	Saturation with O ₂ .	O ₂ capacity (combined).
1923	mm.	mm.	per cent	vol. per cent
Mar. 5	39.5	7.2	7.5	20.95
	38.8	4.2	6.0	
	35.4	8.7	11.1	
	37.6	8.9	12.4	
Mar. 6	41.9	8.3	15.4	22.0
	44.5	32.4	65.8	
	43.4	24.5	49.6	
	41.2	13.0	19.9	
	36.4	19.9	38.3	
	40.4	40.4	80.2	
	47.5	50.4	86.0	
	37.3	38.5	74.6	
Mar. 7	39.3	43.2	77.0	20.67
	39.8	40.0	74.6	
Mar. 10	41.8	44.2	83.0	
Oct. 10	37.0	47.3	84.1	
	40.0	59.2	89.9	
	39.6	59.3	91.5	
	41.3	76.8	95.3	
	37.6	83.7	95.3	
	36.4	86.8	96.0	
	40.8	106.8	100.0	
Oct. 12	42.0	89.5	98.5	21.1
	45.9	98.0	100.0	
	45.0	81.7	96.2	
Oct. 13	41.8	88.8	96.9	
	41.9	99.0	99.0	

Oxygen Dissociation Curve of Blood at a CO₂ Tension of 40 Mm. of Hg.

For the sake of brevity attention in these remarks will be confined to the curve of the blood of A. V. B. The variations of the corresponding curve for the blood of G. S. A. may be determined from comparison of the curves. The small differences found between these two curves appear to be beyond the range of error and are doubtless significant. The points as determined on the blood of A. V. B. are given in Table I, and are plotted in Fig. 1. The curve is drawn free-hand through the points as the most

practical method of determining its character. It will be noted that of a total of 66 points, 28 fall above the curve as drawn, 28 fall below, and 10 are on the curve. If the sum of the squares of all the deviations is taken and the mean square deviation determined, it is found that the square root of this value multiplied by 0.673 gives a "probable error" of about 1 per cent. In other words

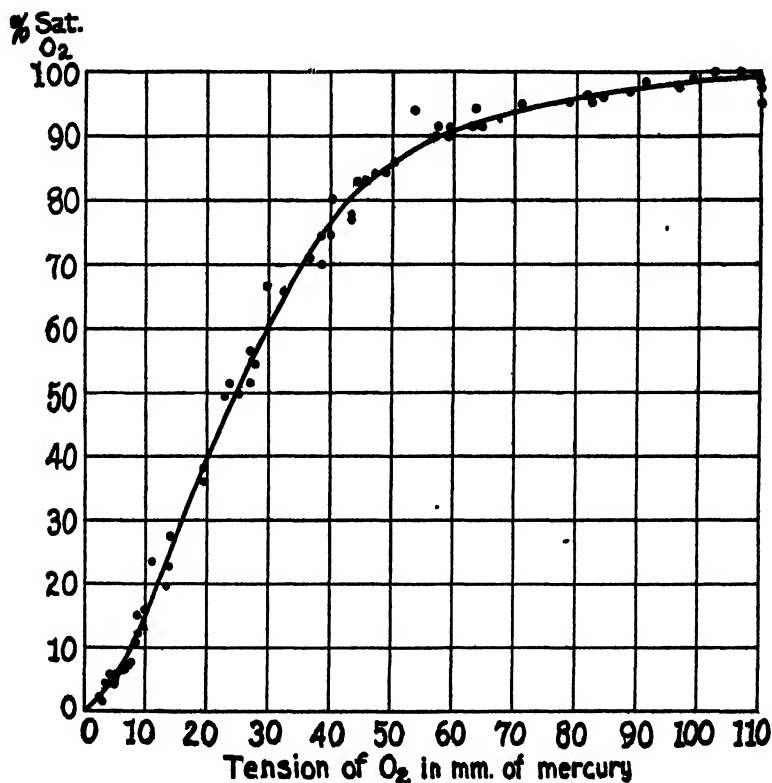


FIG. 1.

taking the tensions of oxygen as being correct and considering the error involved to lie in the percentage saturation of the blood with oxygen, the mean error of all the determinations on the curve is 1 per cent above or below the curve as drawn for all tensions above 10 mm. Below a tension of 10 mm. of Hg the deviation is considerably less than this.

360 O₂ and CO₂ Dissociation Curves of Blood

As contrasted with the curve of Barcroft's blood, the deviation from it of the blood of A. V. B., shown in Fig. 2, expressed in terms of percentage saturation with oxygen at corresponding tensions is as follows:

Hg, mm.....	5	10	20	30	40	50	60	70	80	90	95
Percentage saturation.											
Barcroft.....	1.4	6.0	34.2	58.0	75.0	84.3	90.0	93.0	94.0	95.0	95.4
A. V. B.....	5.3	15.3	39.7	60.2	76.5	85.8	91.4	94.6	96.0	97.0	97.5

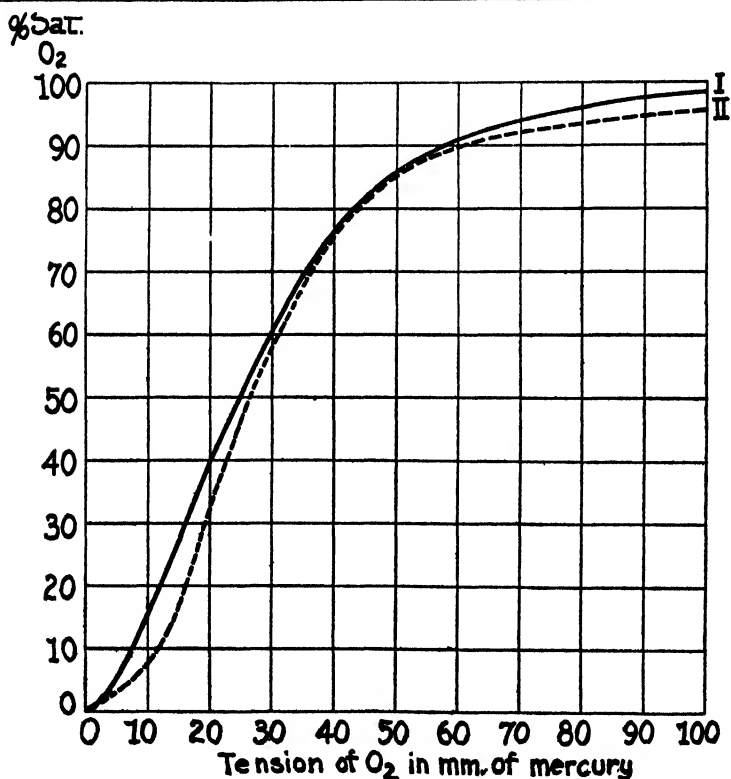


FIG. 2.

The number of points on each curve below a tension of 10 mm. of Hg, the range of deviation of the points from the curves, and the change in form of the new curve as contrasted with the old,

are well shown for the lower range in Fig. 3. The curve and points for Barcroft's blood are taken from a figure published in 1913 (5).

The dissociation curve of the blood of A. V. B. is characterized at low tensions by an S-deflection much less striking than in the case of Barcroft's blood. Attention is also called to the fact that the former curve attains a saturation of 95 per cent at a tension

**% Saturation
with O_2**

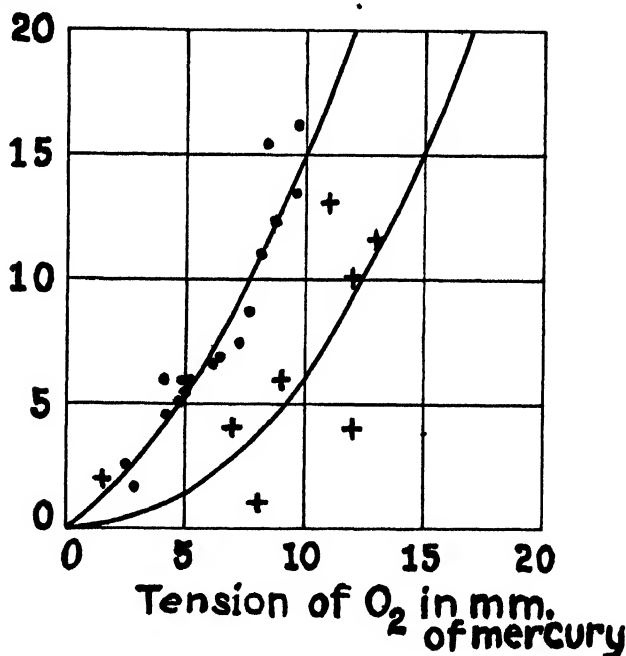


FIG. 3.

of about 70 mm. of Hg, as against 90 mm. for the latter curve. This difference in the upper reaches of the curves may be of some significance from the point of view of diffusion of oxygen from the lungs to the blood.

The data for the blood of G. S. A. are given in Table II and the curve is shown in Fig. 4.

362 O₂ and CO₂ Dissociation Curves of Blood

TABLE II.

Date.	CO ₂	O ₂ (corrected to 40 mm. CO ₂ pressure).	Saturation with O ₂ .	O ₂ capacity (combined).
<i>1922</i>	<i>mm.</i>	<i>mm.</i>	<i>per cent</i>	<i>vol. per cent</i>
July 18	38.6	3.8	7.1	19.6
	41.8	14.8	24.8	
	40.0	22.1	43.3	
	40.0	6.0	6.2	
July 19	41.3	33.5	69.2	19.9
	43.4	44.6	82.2	
	39.4	52.3	88.3	
	41.8	77.6	93.5	
July 22	37.9	53.9	90.2	19.3
	41.8	32.8	65.0	
	41.8	17.8	29.4	
	40.8	70.4	97.0	
	39.3	5.2	3.8	17.3
	33.3	33.0	66.5	
	43.0	41.8	80.0	
	41.8	65.4	91.7	
	39.5	43.3	84.8	18.6
	42.0	84.9	99.8	
	40.5	59.3	91.3	
	39.7	63.2	92.7	
July 25	41.0	89.7	100.0	18.6
	41.4	95.0	97.6	
	42.7	88.0	95.0	
	42.9	95.7	97.6	
<i>1923</i>				
Mar. 17	43.4	4.80	3.5	18.4
	39.0	11.5	12.0	
	40.5	15.6	19.9	
	43.9	5.4	4.7	
	45.9	25.0	42.9	18.9
	38.8	36.0	72.5	
	40.1	41.7	79.2	
	41.7	13.0	16.5	
Mar. 20	41.9	5.5	4.4	17.15
	43.8	15.8	20.0	
	43.8	21.6	34.8	
	41.6	25.4	53.0	
Mar. 22	42.0	4.7	5.0	17.15
	44.7	5.8	5.8	
	42.4	13.2	18.3	
	41.2	16.4	24.0	

TABLE II—*Concluded.*

Date.	CO ₂	O ₂ (corrected to 40 mm. CO ₂ pressure).	Saturation with O ₂ .	O ₂ capacity (combined).
1923	mm.	mm.	per cent	vol. per cent
Mar 30	45.0	25.2	48.7	17.3
	41.3	29.6	56.2	
	39.7	39.5	76.5	
	42.6	79.4	97.3	
	42.9	82.8	98.0	
	41.7	88.7	100.0	
	40.6	62.6	93.7	
	38.4	49.5	88.5	

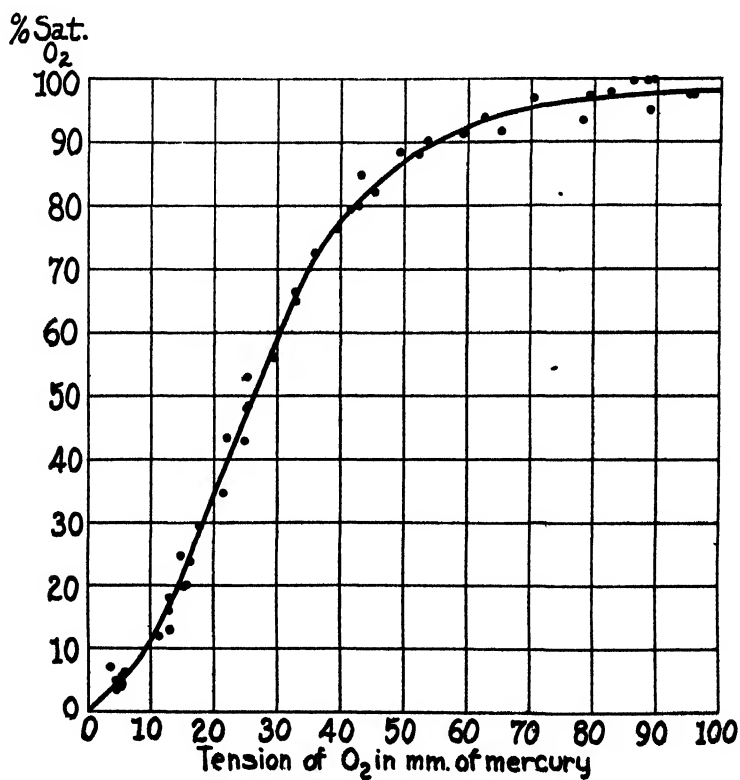


FIG. 4.

364 O₂ and CO₂ Dissociation Curves of Blood

TABLE III.

Date.	CO ₂	O ₂	Saturation with O ₂ .	O ₂ capacity (combined).
1923	mm.	mm.	per cent	vol. per cent
Mar. 13	0.57	16.2	69.2	20.4
	2.7	26.3	88.8	
	5.1	37.0	96.0	
	2.8	12.9	48.8	
	2.6	5.0	13.0	
	2.9	37.8	96.0	
	3.7	11.5	47.2	
	2.7	15.9	60.4	

TABLE IV.

Date.	CO ₂	O ₂ (corrected to 20 mm. CO ₂ pressure).	Saturation with O ₂ .	O ₂ capacity.
1923	mm.	mm.	per cent	vol. per cent
May 4	20.5	4.5	5.4	19.85
	23.8	13.3	30.8	
	23.8	28.5	67.7	
	22.9	31.2	73.8	
	21.5	43.4	85.3	
	19.6	22.2	55.5	
	18.8	47.2	88.7	
	24.1	39.0	83.2	
	18.0	64.5	97.0	
May 7	19.4	72.2	98.7	18.7
	20.3	37.0	86.0	
	19.6	32.8	79.3	
	20.1	61.2	97.0	
	19.3	18.9	41.8	
May 8	22.5	41.8	86.0	18.68
	22.5	56.0	93.0	
	19.9	12.4	24.3	
	19.4	21.3	53.5	
	20.2	65.6	97.2	
	19.0	35.0	83.7	
	19.8	5.6	7.7	

Oxygen Dissociation Curves of Blood at CO₂ Tensions of 3, 20, and 80 Mm. of Hg.

The 3 Mm. Curve.—In the case of this curve the points are few in number but their accuracy is sufficiently great to establish the

curve as drawn free-hand through them. The data comprise Table III.

The 20 Mm. Curve.—Of 21 points determined on this curve 6 fall above the free-hand curve, 9 fall below it, and 6 points are on the curve. The points are listed in Table IV.

TABLE V.

Date.	CO ₂	O ₂ (corrected to 80 mm. CO ₂ pressure).	Saturation with O ₂ .	O ₂ capacity (combined).
1923	mm.	mm.	per cent	vol. per cent
Mar. 14	80.3	14.4	9.8	
	77.8	17.8	28.0	
	76.6	44.9	75.3	
	88.0	29.6	43.2	
	83.4	65.4	87.0	
	76.2	97.8	93.5	
Mar. 16	85.4	7.1	4.5	21.1
	90.4	17.3	13.0	
	92.0	33.7	45.0	
	78.5	46.4	71.8	
	83.2	62.0	87.6	
	85.2	103.6	98.0	
Mar. 31	89.7	20.8	23.8	20.55
	87.4	6.5	4.7	
	91.2	12.7	14.7	
	73.2	24.5	28.0	
	81.2	42.4	59.3	
	75.0	50.7	76.0	
Apr. 3	72.2	56.5	85.0	21.5
	81.0	101.2	98.3	
	73.3	20.9	29.0	
	89.3	24.9	39.0	
	78.6	28.3	41.0	
	86.0	30.9	47.0	
May 12	86.2	35.2	55.1	18.7
	85.2	17.4	18.3	
	71.3	17.4	17.5	
	75.4	57.2	82.7	
	76.8	61.7	86.3	
	71.2	57.0	84.9	

The 80 Mm. Curve.—Of 30 points on this curve, 10 fall above it, 14 below, it, and 6 fall upon it. The data are given in Table V.

366 O₂ and CO₂ Dissociation Curves of Blood

No great degree of accuracy is claimed for these three curves owing to the insufficient number of points by which they were determined. In the experimental work more difficulty was experienced in getting consistent results when the tension of CO₂ was at 80 mm. of Hg than at the lower tensions. Whether the equilib-

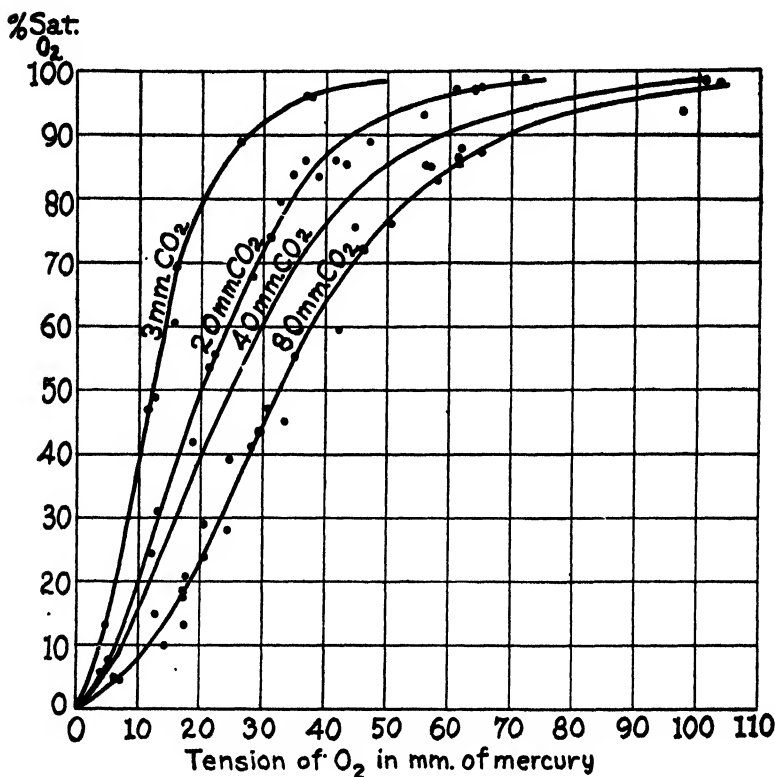


FIG. 5.

rium of the system is more unstable at the higher tension of CO₂ or not is a matter for future investigation. The curves are shown in Fig. 5, and may be compared with a similar figure in Barcroft's book.¹

¹ Barcroft (1), p. 65.

Hill's Equation Applied to the Oxygen Dissociation Curve of Blood.

When the oxygen dissociation curves of blood are plotted in the usual way, y , the ordinate, being the fraction of HbO_2 , and x , the abscissa, the tension of oxygen in mm. of Hg, it will be seen that our curves and Barcroft's are nearly the same in shape and correspond roughly to the curve of Hill's equation. The fit in

TABLE VI.

$\log \frac{y}{1-y}$	$\log x_1$	$\log x_{10}$	$\log x_{40}$	$\log x_{60}$	$\log x_{100}(\text{G.S.A.})$
-1.28	0.38	0.60	0.65	0.86	0.72
-0.95	0.60	0.83	0.87	1.07	0.98
-0.60	0.81	1.01	1.08	1.26	1.16
-0.37	0.93	1.12	1.21	1.37	1.26
-0.18	1.02	1.22	1.31	1.44	1.35
0.00	1.10	1.30	1.39	1.51	1.42
0.18	1.16	1.38	1.47	1.58	1.48
0.37	1.23	1.46	1.55	1.65	1.55
0.60	1.31	1.54	1.64	1.73	1.63
0.95	1.42	1.65	1.76	1.84	1.74
1.28	1.54	1.74	1.86	1.94	1.83

TABLE VII.

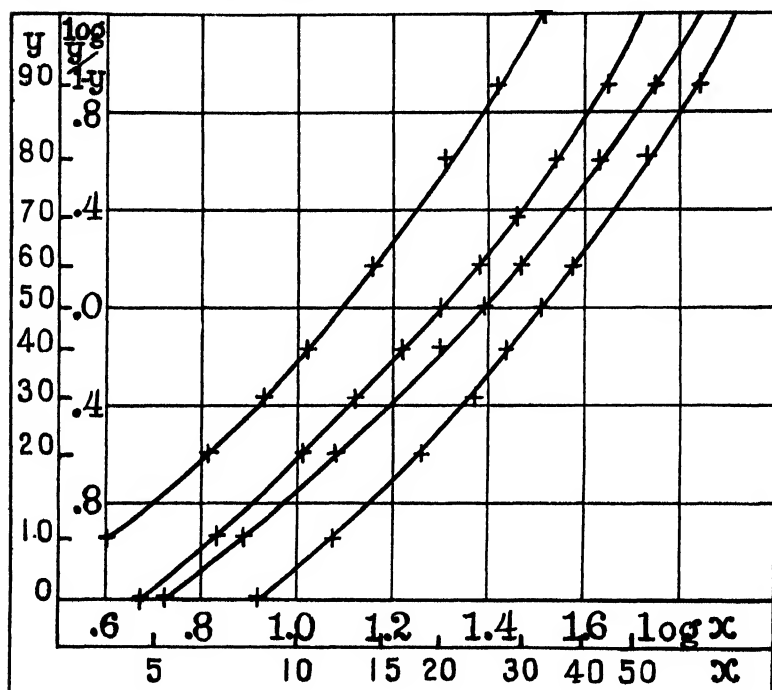
y	x at 3 mm. CO_2	x at 20 mm. CO_2	x at 40 mm. CO_2	x at 80 mm. CO_2	x at 40 mm. CO_2 (G.S.A.)
5	2.4	4.0	4.5	7.3	5.3
10	4.0	6.7	7.4	11.7	9.6
20	6.4	10.2	12.0	18.4	14.5
30	8.5	13.2	16.1	23.2	18.4
40	10.5	16.6	20.2	27.7	22.2
50	12.5	20.1	24.5	32.5	26.4
60	14.3	24.0	29.6	37.7	30.4
70	17.0	29.0	35.6	44.3	35.1
80	20.4	34.6	44.1	53.4	42.3
90	26.5	44.2	58.2	69.2	55.2
95	34.8	54.5	72.5	87.0	67.5

the former set of curves, however, is not exact, but the method of plotting makes it difficult to discuss the nature of the deviation. This can be brought out by plotting the relations involved in logarithmic form, according to Hill's theory, as has recently been done by Brown and Hill (6).

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$$\begin{aligned}
 (\text{Hb}) \times (\text{O}_2)^n \times K &= (\text{HbO}_2) \\
 (1 - y) \times (x)^n \times K &= y \\
 \log (1 - y) + n \log x + \log K &= \log y \\
 n \log x + \log K &= \log y - \log (1 - y)
 \end{aligned}$$

Hence if we calculate $\log \frac{y}{1-y}$ and $\log x$ for each point, and plot the new variables with $\log x$ as abscissa, the result should be a straight line if Hill's equation describes the new curves. In Table



VI we have calculated the logarithmic variables from the values of x and y in Table VII. The results are plotted in Figs. 6 and 7. It will be seen that the lines curve upwards. If Hill's theory applied over the whole range, the lines would be straight. An empirical formula covering the entire range of tensions would have to include one more term.

$$n \log x + \log K + b (\log x) = \log y - \log (1 - y)$$

Bock, Field, and Adair

If the physiological range is considered alone, Hill's formula is accurate enough for all practical purposes, provided the values of n and K are used which apply to this range. These calculations are tabulated in Table VIII. B_3 , B_{20} , B_{40} , and B_{80} are from the oxygen dissociation curves of A. V. B., at 3, 20, 40, and 80 mm. of CO_2 , and A_{40} , that of G. S. A. at 40 mm. of CO_2 . These observa-

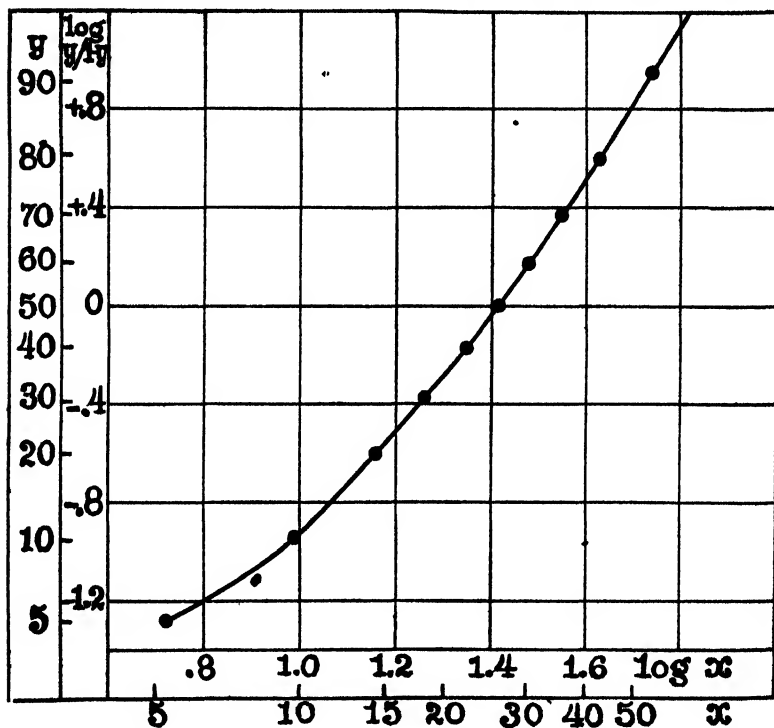


FIG. 7.

tions confirm Barcroft's finding that change of CO_2 tension alters K rather than n , but they show that n is not constant over the range from full reduction to full oxidation at constant CO_2 tension. The mean values, $n = 2.33$, upper range, and $n = 2.0$, lower range, have been used in calculating K .

It will be seen that the value of n for the upper range of the blood of A. V. B., 2.33, agrees with that of Douglas, and the value of n for the upper range of the blood of G. S. A., 2.55, agrees with that

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of Barcroft. For some time we suspected that the curve for the blood of G. S. A. might be wrong as there were fewer points, but further observations forced us to conclude that there is a difference in the form of the two oxygen dissociation curves. There are other published curves which agree with ours in indicating a low value of n at low saturations. For example, in the curve of J. S. Haldane (7), the value of n for the upper range is 2.2 and for the lower range $n = 2.0$.

The deviation of our curves from those described by Hill's equation is significant. Since the curve described by this equation fitted the experimental points determined by Barcroft, the latter ascribed physical meanings to empirical constants and upon this

TABLE VIII.

y	$\log \frac{y}{1-y}$	$\log x, B_1$	$\log x, B_{20}$	$\log x, B_{40}$	$\log x, B_{60}$	$\log x, A_{40}$
90.91	1.0	1.52	1.72	1.84	1.92	1.81
50.0	0.0	1.10	1.30	1.38	1.51	1.42
9.09	-1.0	0.58	0.8	0.86	1.05	0.97
$n, 50-90$		2.38	2.38	2.27	2.3	2.53
$n, 9-50$		1.92	2.0	1.92	2.17	2.22
$\log \frac{1}{K} 50-90$		367	1,070	1,640	3,270	3,930
$\log \frac{1}{K} 9-50$		158	398	575	1,050	315

basis the Barcroft-Hill (1) aggregation theory of hemoglobin was constructed. Our data do not conform to Hill's equation, but we have no alternative theory to offer as to the mechanism by which the combination of hemoglobin and oxygen in blood is attained.

Carbon Dioxide Dissociation Curves.

The form of the carbon dioxide dissociation curves of fully oxygenated and fully reduced whole blood was determined by Christiansen, Douglas, and Haldane (8). These workers suggested that the greater capacity of reduced blood to combine with CO₂ might be due to the fact that reduced hemoglobin was less acid than oxyhemoglobin. That this is probably the case has subsequently been shown by Hasselbalch (9), Parsons (10), and L. J.

Henderson (11). The significance of the form of the CO_2 curve has been discussed by many writers and need not be dwelt on here. In the laboratory this curve is commonly used for two purposes. In the first place, the level of the curve at any given tension of carbonic acid shows the amount of base available for the transport of CO_2 . That the quantity of this base in a given individual in health is fairly constant over long periods of time was shown by Christiansen, Douglas, and Haldane (8). In the case of the blood of A. V. B. variations from day-to-day of 1 or 2 volumes per cent

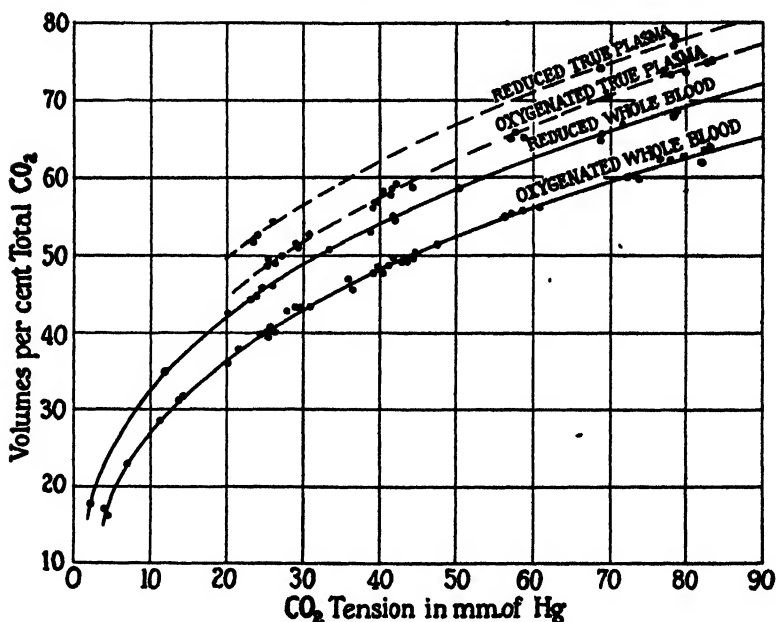


FIG. 8.

only of total CO_2 were encountered over a period of several months. There can be no doubt, however, from a study of many curves in the literature of normal individuals that the level of the curve at any tension of CO_2 , *e.g.* 40 mm., may vary from individual to individual, although it has recently been assumed by Dautrebande, Davies, and Meakins (12) that some of these curves are in error because they do not coincide with that of the blood of Haldane.

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TABLE IX.

Date.	CO ₂	O ₂	Total CO ₂ .	Total CO ₂ of true plasma.	O ₂ capacity.
<i>1888</i>	<i>mm.</i>	<i>mm.</i>	<i>vol. per cent</i>	<i>vol. per cent</i>	
Apr. 4	20.0	Air.	36.1		21.53
	25.7	"	40.7		
	60.7	"	56.2		
	13.7	"	31.2		
Apr. 5	44.1	"	49.7		21.0
	24.4	"	39.7		
	47.5	"	51.4		
	56.4	"	55.0		
	11.2	"	28.6		
	73.8	"	59.8		
	36.4	"	45.5		
	4.3	"	16.2		
Apr. 10	42.8	"	49.1		
	41.1	"	48.7		
	43.4	"	49.3		
	35.8	"	47.0		
	72.4	"	60.2		
Apr. 11	21.5	"	37.7		
	41.6	"	49.7		
	40.3	"	47.8		
	27.6	"	42.8		
	14.1	"	31.7		
	44.5	"	50.0		
Apr. 12	42.0	5.0	54.6		
	20.2	3.0	42.4		
	33.3	4.7	50.7		
	50.3	5.4	58.8		
	11.9	6.0	34.9		
Apr. 15	2.2	5.1	17.6		
	71.7	3.3	67.0		
	41.6	6.0	55.1		
	38.3	5.8	53.1		
	24.5	6.1	45.7		
	4.2	Air.	17.1		
	39.8	"	48.5		
May 23	29.2	"		51.1	19.5
" 28	29.4	"	43.3		
" 29	78.0	"	62.4	73.5	19.5
" 31	41.35	"		58.0	19.0
	42.2	"		59.4	
June 1	39.7	"		57.0	18.7

TABLE IX—*Concluded.*

Date.	CO ₂	O ₂	Total CO ₂ .	Total CO ₂ of true plasma.	O ₂ capacity.
<i>1923</i>	<i>mm.</i>	<i>mm.</i>	<i>vol. per cent</i>	<i>vol. per cent</i>	
June 4	44.2	Air		58.8	18.2
" 5	40.3	"		58.3	
	82.0	"	62.0		
June 6	69.0	9.0	65.7		
July 25	77.4	Air.	62.8	73.9	
Oct. 1	27.0	"		49.9	19.5
	41.5	"		58.7	
	57.2	"	55.4	65.2	
	7.1	"	23.0		
	30.7	"	43.1	52.8	
Oct. 2	69.8	"		70.5	
	57.7	"		65.9	
	39.1	"	47.7	56.2	
	29.1	"	43.1	51.3	
	58.7	"	55.7	65.2	
Dec. 10	25.4	"	39.5	49.5	
	83.1	"	64.25	75.1	
	26.2	"	40.0	48.9	
	25.2	"	39.7	48.7	
	79.8	"	62.75	73.75	
	82.8	"	63.5	75.0	
Dec. 11	78 65	4.0	68.9	78.3	
	25.9	5.1	46.4	54.5	
	68.8	4.1	65.3	74.4	
	23.9	2.0	44.9	52.6	
	78.5	4 3	68.5	77.2	
	23 3	2.0	44.45	51.9	

- In the second place, the curve may be used in determining the amount of additional CO₂ that will be taken up by the same blood when it is reduced as compared with that of the fully oxygenated state. If one disregards the small change in (H⁺), this added amount at any given tension of CO₂ represents the amount of base transferred from combination with hemoglobin to that combined with carbonic acid. The phenomenon takes place partially in the body with every cycle of the blood through the lungs, the mixed venous blood of normal resting individuals being about 25 to 30 per cent reduced. In the case of the blood of A. V. B. the average difference in the amount of CO₂ between the curves

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of blood fully oxygenated and fully reduced at tensions of CO₂ from 40 to 80 mm. of Hg is 6.3 volumes per cent. The data upon which the curves of whole blood shown in Fig. 8 were constructed are given in Table IX. In the laboratory it is difficult to obtain complete reduction of blood in the tonometers under the conditions of the experiment, but in the case of every point determined for reduced blood correction has been made for the amount of oxygen

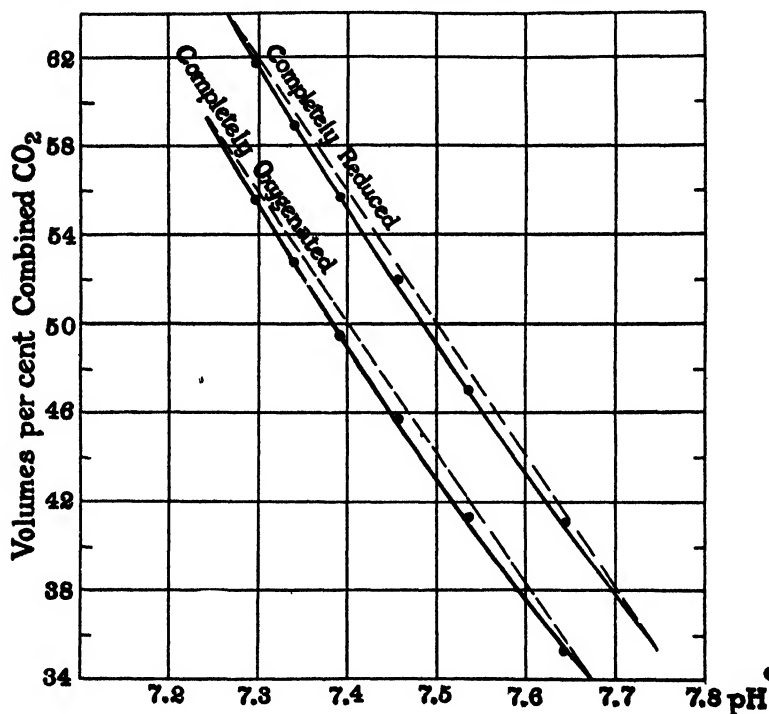


FIG. 9.

present by reading off the percentage saturation with oxygen on appropriate oxygen dissociation curves at the tension of oxygen found to be present in each tonometer.

Straight Line Relationship of the CO₂ Curve.

Attempts have been made by Warburg (13) and Van Slyke, Austin, and Cullen (14) to express the carbon dioxide absorption curve as a straight line when plotted with pH against bicarbonate.

Peters and his associates (15) have shown that this relationship only roughly approximates a straight line over short ranges of pH and that for the usual range covered by the curve the deviation from a straight line at a tension of 40 mm. of Hg is ± 1.26 per cent. After study of a considerable mass of data on the subject, Peters showed that when the curve is plotted as log

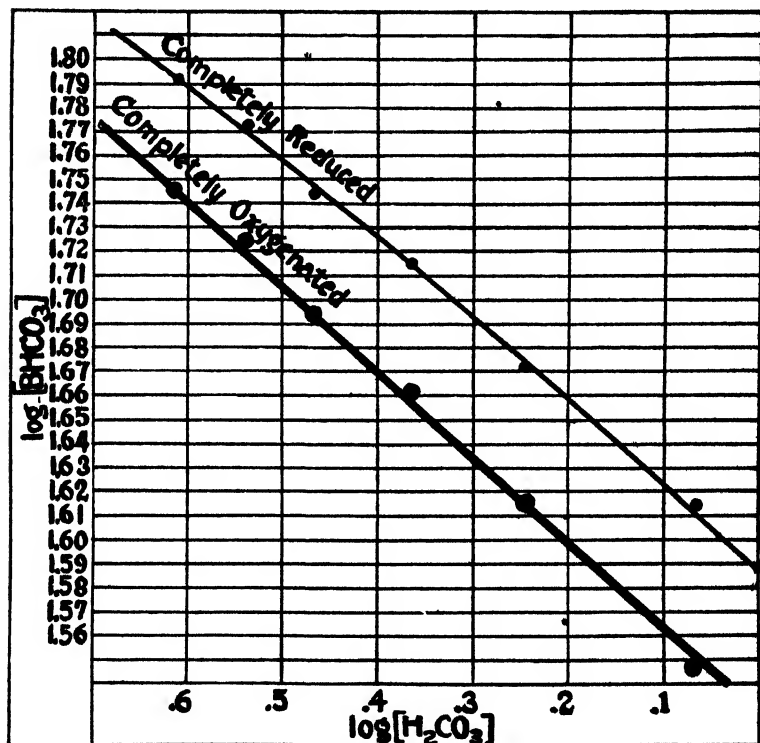


FIG. 10.

(H₂CO₃) against log (BHCO₃) the result is more nearly a straight line. The matter was tested in the case of the blood of A. V. B. and the results are shown in Figs. 9 and 10. H₂CO₃ was calculated following the data of Van Slyke, Wu, and McLean, (16), and pH with Hasselbalch's formula, using 6.16 for the value of pK₁, this being the apparent value of pK₁ as calculated from data given in the following paper. The log (H₂CO₃)- log (BHCO₃)

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relationship is approximately a straight line for fully oxygenated blood and permits, as Peters has pointed out, determination of the entire CO₂ curve by extrapolation if two points have been experimentally determined.

True Plasma Carbon Dioxide Dissociation Curves.

There are very few data in the literature for true plasma curves determined on plasma from fully oxygenated and fully reduced blood. The most complete curves are those of the blood of J. J. given by Joffe and Poulton (17). The present data are published chiefly to supplement those now available. The data are included in Table IX and the curves are plotted in Fig. 8.

At a CO₂ tension of 40 mm. of Hg, the difference in the CO₂ content between oxygenated whole blood and oxygenated true plasma is 9.5 volumes per cent; at 80 mm. it is 11.6 volumes per

TABLE X.

	Total CO ₂ of whole blood.	Total CO ₂ of corpuscles.	Total CO ₂ of plasma.
	vol. per cent	vol. per cent	vol. per cent
Arterial blood.....	48.0	13.5	34.5
Venous blood.....	53.0	15.6	37.4
Difference.....	5.0	2.1	2.9

cent. For the reduced blood and plasma the corresponding figures are 7.8 and 8.9 volumes per cent. The absence of oxygen obviously reduces the amount of CO₂ taken up by reduced true plasma by 1.7 volumes per cent at a tension of 40 mm. of Hg, probably because a somewhat greater proportion of CO₂ is carried in the corpuscles of reduced blood than in those of oxygenated blood. This follows from the more alkaline character of reduced hemoglobin.

It is shown in the following paper that the tension of CO₂ in the arterial blood of A. V. B. is approximately 40 mm. of Hg, that of the mixed venous blood is 47 mm. of Hg, that the saturation with oxygen of the arterial blood is 95.5 per cent, and that of the mixed venous blood is about 65 per cent. The average oxygen capacity is 20 volumes per cent and the corpuscle volume is 40 per cent. If, for the sake of simplicity, the curve for fully

oxygenated blood is taken to represent the arterial blood, and allowance is made for the reduction of the mixed venous blood, it is possible to read from the curves of Fig. 8 the amount of CO_2 contained in the arterial and mixed venous blood and the amount of CO_2 in the true plasma separated from the blood at the above tensions. The amount of CO_2 taken up in a respiratory cycle by both corpuscles and plasma can then be calculated. The figures are given in Table X. As shown also in the following paper it thus appears that the transport of CO_2 during a respiratory cycle is accomplished by both corpuscles and plasma in the ratio of approximately 40 to 60 per cent.

SUMMARY.

1. The oxygen dissociation curves determined at a CO_2 tension of 40 mm. of Hg for the blood of two normal subjects are given. Similar curves at CO_2 tensions of 3, 20 and 80 mm. of Hg for the blood of one of the subjects are also presented.

2. It is shown that these oxygen dissociation curves do not conform to Hill's formula.

3. Carbon dioxide dissociation curves on fully reduced and fully oxygenated blood as well as the corresponding true plasma curves are given.

4. Both corpuscles and plasma transport carbon dioxide; the former carrying 40 per cent of the total and the latter, 60 per cent.

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BLOOD AS A PHYSICOCHEMICAL SYSTEM. II.*

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In the pages which follow an attempt has been made to describe the respiratory mechanism of normal human blood. The treatment of this subject consists of three parts: first, a general or synthetic description, in the form of a nomogram, of all the physicochemical factors which are known to be involved in the complex equilibrium of the blood; secondly, a detailed or analytic description, with the aid of many contour line charts, of those relations between the several factors which conveniently lend themselves to separate consideration; and thirdly, an investigation of the implications of the diffusion theory concerning the respiratory exchanges in lung and tissue capillaries.

Since respiratory phenomena are alone in question, we shall be concerned, as usual, with blood which is approximately in a steady state. Such blood is liable to no other variations in the concentrations of its ultimate components than the cyclic respiratory changes in oxygen and carbonic acid. At any point of the circulation, for example in the pulmonary artery or vein, its composition is in all respects approximately constant. The customary methods of studying blood in the laboratory, which involve external variations in oxygen and carbon dioxide tensions exclusively, are easily seen to be consistent with this restriction.

* This paper is study No. 38 in a series of studies of the physiology and pathology of the blood from the Harvard Medical School and allied Hospitals, the expenses of which have been in part defrayed by the Proctor Fund for the study of chronic diseases.

In our first discussion of the present subject (1) it was shown that all external changes in oxygen or carbon dioxide tension are invariably accompanied in blood, not only by changes in free and combined oxygen and carbonic acid concentrations, but also by changes in hydrogen ion concentration and in the distribution of chlorides between cells and plasma. These changes are all parts of a process by which a definite physicochemical equilibrium is readjusted when the equilibrium has been disturbed. The known phenomena, commonly represented by oxygen dissociation curves, carbon dioxide dissociation curves, the equation of the acid-base equilibrium, and so on, are but partial aspects of this process, which indeed has many other partial aspects, such as the relation between oxygen or carbon dioxide tension, hydrogen ion concentration and chloride distribution, and the like. After these facts had been established, it was possible to represent the complete equilibrium, as far as six variables are concerned, by means of a Cartesian nomogram. It is not too much to say that, once clearly stated, the conclusion of this investigation is obvious, alike from the standpoint of the physical chemist, and from that of the physiologist.

The original nomogram does not, however, give a complete description of the entire physicochemical equilibrium, since it leaves out of account the movement of water between cells and plasma. But at this point the necessary experimental data were not at hand. It was indeed believed, in accordance with theoretical considerations long ago set forth by Spiro and Henderson (2), that the differences between changes in serum chloride concentration and simultaneous changes in serum bicarbonate concentration, when hydrogen ion concentration is held constant, must be due, at least in greater part, to the movement of water between plasma and corpuscles. Yet, in the absence of the necessary data, proof was at the moment impossible.

This investigation raised a large number of questions, and, since the study of all these could not be continued at Harvard, a division of labor was arranged whereby the chloride problem, upon which F. C. McLean was then working here, and the related problem of water distribution, should be investigated in the Hospital of The Rockefeller Institute by Van Slyke and McLean. This work has now been brought to a brilliant conclusion, and

has resulted in the paper by Van Slyke, Wu, and McLean (3), which, at least with a satisfactory approximation to accuracy, seems to have settled the problem. It now appears that the theory of Spiro and Henderson (2) concerning water and chloride distribution as a function of buffer action of proteins, of alkali reserve, in the restricted sense of the term, and of osmotic pressure, is valid. This theory, suitably extended to take account of two later discoveries, the influence of oxygenation upon the acidity of hemoglobin (4, 5, 6), and the applicability of the Gibbs-Donnan equation to the distribution of anions between cells and serum (3, 7), completely accounts for all the hitherto uncertain factors of the blood equilibrium with such exactness as present experimental methods permit. It is a well founded conclusion that all the known phenomena of the respiratory cycle in blood may be completely described with a good approach to accuracy by taking into account the six variables of our earlier nomogram plus that one, the water distribution, of which the treatment had to be postponed for lack of data.

The theoretical importance of Van Slyke, Wu, and McLean's paper is, however, greater than this, for we now possess a unified and at the same time quantitative mathematical treatment of a subject which hitherto has existed only in fragments. Moreover, the discussion is cast in such a form that new discoveries or other modifications can be added without invalidating conclusions already reached. Thus, for example, if it should become necessary to take account of the surface tension of the red cells in addition to osmotic pressure, in order to define with greater accuracy the water equilibrium, this could be done without difficulty.

This investigation also goes far toward establishing the thesis, which is implied in the paper of Spiro and Henderson (2) and in the two papers of Henderson (1, 6), that the components of the system of which consideration is necessary and sufficient for the proper approximate treatment of the respiratory changes are H_2O , O_2 , CO_2 , serum base, cell base, serum protein, cell protein, and Cl and H ions.

In the present paper we shall, for convenience, fix our attention on the following seven variables: free oxygen, total oxygen, free carbonic acid, total carbonic acid, hydrogen ion concentration

of serum (expressed as pH), the volume of the corpuscles, and the ratio of the concentrations of anions within and without the cell (the r of Van Slyke, Wu, and McLean). Beyond the consideration of the varying distribution of water, expressed as change of volume, these represent merely slight modifications in the arbitrary choice of variables, of which, as we shall see, there are many others at our disposal. They do not in any sense change the nature of the problem or of its treatment, and, as will appear, treatment and results are in all respects consistent with the original nomogram (1).

I.

The construction of a complete nomogram may be conveniently begun with the help of the familiar oxygen and carbon dioxide dissociation curves. For the present purpose we have at our disposal the oxygen dissociation curves of the blood of A.V.B. at 3, 20, 40, and 80 mm. of CO_2 tensions, respectively (8). These have been constructed with the aid of more than 90 experimental determinations. The curves yield Table I.

We also possess carbon dioxide dissociation curves for the reduced and oxygenated blood of A.V.B. drawn with the aid of about 60 experimental determinations (8). These curves yield Table II.

From Table II, Fig. 1 has been constructed. Here values of total oxygen are plotted as abscissæ, values of total carbonic acid as ordinates, while values of carbon dioxide tension appear as isopleths or contour lines. These lines are almost exactly straight, because at constant hydrogen ion concentration equal changes in oxygenation are accompanied by equal changes in base bound by hemoglobin (1, 9). The values of total oxygen have been spaced in units of percentage by volume (100 per cent $\text{HbO}_2 = 20$ volumes per cent) in order to obtain the same values for the scales of ordinates and of abscissæ.

The next step consists in applying the data of Table I to Fig. 1, as follows: Beginning with the case $\text{O}_2 = 5$ mm., we select the pair of values $\text{CO}_2 = 3$ mm. and $\text{HbO}_2 = 13.5$ per cent of complete saturation, or 2.7 volumes per cent of total O_2 , 20 volumes per cent being assumed to represent 100 per cent saturation. We find

the point on Fig. 1 where the abscissa corresponding to this value of total oxygen cuts the contour line corresponding to $\text{CO}_2 = 3$ mm. and mark the point. This process is then repeated for

TABLE I.
Effect of CO_2 Tension on Oxygen Curves of Human Blood.

O_2 tension. mm.	HbO ₂ Oxygen saturation of blood at following CO_2 tensions.			
	$\text{CO}_2 = 3$ mm.	$\text{CO}_2 = 20$ mm.	$\text{CO}_2 = 40$ mm.	$\text{CO}_2 = 80$ mm.
	saturation per cent	saturation per cent	saturation per cent	saturation per cent
5	13.5	6.8	5.5	3.0
10	38.0	19.5	15.0	8.0
20	77.6	50.0	39.0	26.0
30	92.0	72.2	60.6	44.8
40	96.7	87.0	76.0	63.5
50	98.5	93.3	85.5	76.9
60	100	96.3	90.5	85.0
70	100	98.0	94.0	90.3
80	100	99+	96.0	93.7
90	100	100	97.5	95.7
100	100	100	98.6	97.1

TABLE II.
 CO_2 Absorption Curves of Oxygenated and Reduced Human Blood.

CO_2 tension. mm.	Total CO_2 content.	
	Oxygenated blood (HbO ₂ = 100 per cent).	Reduced blood (HbO ₂ = 0 per cent).
	vol. per cent CO_2	vol. per cent CO_2
3	14.0	19.5
10	26.8	32.5
20	36.5	42.4
30	43.0	49.1
40	48.0	54.3
50	52.2	58.6
60	56.2	62.5
70	59.7	65.9
80	63.0	69.1

the other pairs of values of CO_2 tension and total oxygen corresponding to $\text{O}_2 = 5$ mm. and the four points are joined by a smooth curve. This curve is the isopleth or contour line for

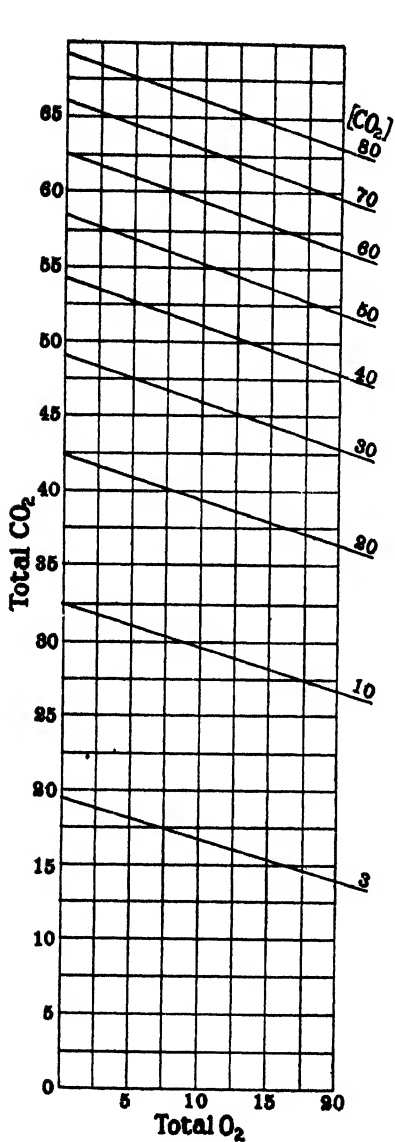


FIG. 1.

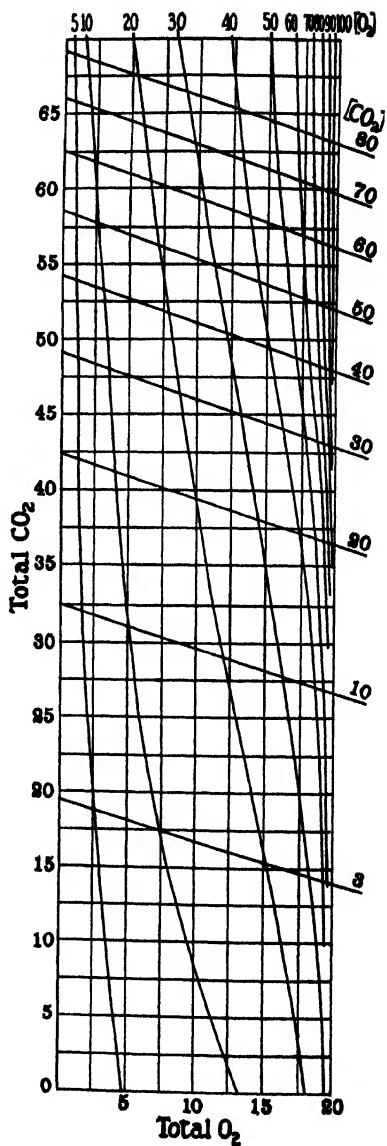


FIG. 2.

$O_2 = 5$ mm. and is so marked. The process is now repeated in turn for $O_2 = 10, 20, \dots, 100$ mm., with the result represented by Fig. 2.

This is a Cartesian nomogram which completely illustrates the conditions of equilibrium between free and total oxygen and free and total carbonic acid, incompletely expressed by the dissociation curves. Because the Cartesian coordinates are values of total oxygen and total carbonic acid, respectively, while the contour lines represent the true physiological variables, it is in some respects the most useful of all representations of the blood equilibrium. These advantages will appear in the sequel.

At this stage it will be convenient to undertake a transformation to an alignment chart, or nomogram, of the type invented by d'Ocagne (10). The necessary construction for such a transformation (Fig. 3) is as follows (11):

Let Ox and Oy be the axes of a Cartesian nomogram and KL any straight line. Draw two parallel axes Au and Bv . Now read the Cartesian coordinates, x_1, y_1 , and x_2, y_2 , of any two points, say p_1 and p_2 , of the line KL . On Au lay off (taking account of sign) the distance $AM = x_1$ and on Bv the distance $BP = y_1$. Join MP . On Au lay off the distance $AQ = x_2$ and on Bv the distance $BN = y_2$. Join NQ . Then C , the point of intersection of MP and NQ , is the correlative of the line KL . If KL is one of several contour lines, points corresponding to the others may be found by the same method and, upon joining all these points, a scale of values of the variable, z , corresponding to the contour lines, is obtained.

This process may be repeated for any other family of contour lines, corresponding to values of any other variable, w , on the same Cartesian background. Graduation of Au and of Bv to represent values of the variables x and y completes the construction.

The chart is read with the help of a thread stretched across the scales. It has the property that the values intercepted on the scales by any straight line are simultaneous values of the several variables. This is obvious, because, on such a chart, a straight line corresponds to a point on a Cartesian nomogram. Therefore the intercepts of the line on the scales correspond to the values of the variables represented by the scales at the point of the

Cartesian nomogram which is the correlative of the straight line in question.

D'Ocagne's method has been widely applied and many expositions of the subject are now available. For further information his own treatise (11) or that of Lipka (12) may be consulted.

It is easy to see that this method is only applicable to Cartesian nomograms on which the contour lines are straight or may be so regarded without serious error. In the present case, however,

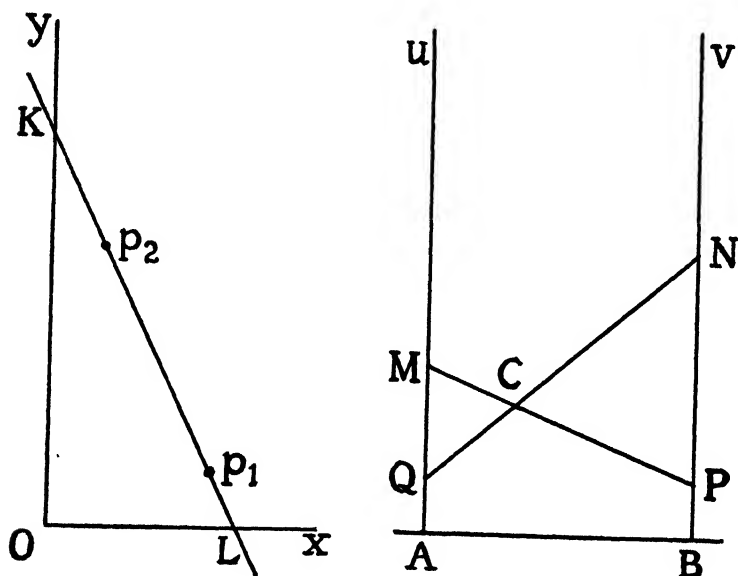


FIG. 3.

the curvature of the contour lines of oxygen tension is considerable. Nevertheless, by confining our attention to the region of the chart where the CO_2 tensions fall within the physiological range, it is possible to replace the oxygen contour curves by straight lines, without introducing serious error. We may then proceed to the transformation. When completed this results in the four scales marked HbO_2 , O_2 , CO_2 , and total CO_2 of Fig. 4.

The scales of Fig. 4 marked Vol. , r , and pH , complete the nomogram. For their construction we have made use of further

experimental data. These consist of: (1) measurements of the total CO_2 content of true plasma of specimens of blood of A.V.B., in which the O_2 tensions, total CO_2 , and CO_2 tensions of the whole blood were known (Table III); and (2) measurements of the indices of refraction of samples of true plasma of the blood of A.V.B. for which O_2 and CO_2 tensions were known (Table IV).

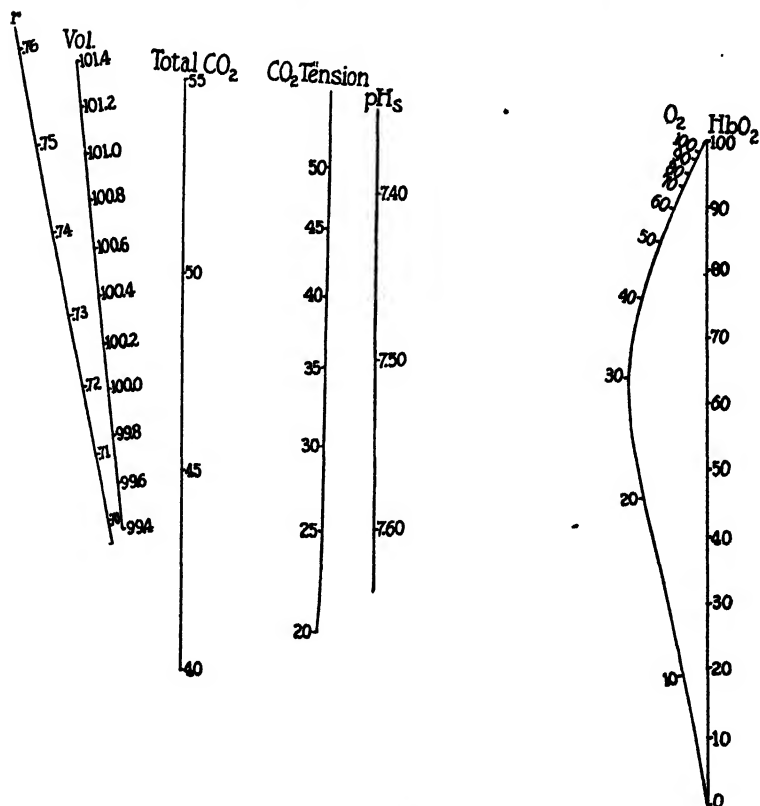


FIG. 4.

From the determinations of indices of refraction of serum, after slight corrections for fluctuations from day to day, the volumes of the corpuscles were calculated. These volumes were expressed as percentages of the cell volume when $\text{O}_2 = 80$ mm. and $\text{CO}_2 = 39$ mm., which was found by numerous hematocrit readings to average 40 per cent of the total blood volume. At this

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point cell water constituted 65 per cent of total cell volume and serum water 91.5 per cent of total serum volume. This calculation calls for no comment, since, for the conditions of the experiment, index of refraction varies inversely with water content.

TABLE III.

CO ₂	O ₂	Total CO ₂ of whole blood.	Total CO ₂ of true plasma.
<i>mm.</i>	<i>mm.</i>	<i>vol per cent</i>	<i>vol. per cent</i>
29.2	Air.		51.1
78.0	"	62.4	73.5
39.7	"		57.0
44.2	"		58.8
40.3	"		58.3
27.0	"		49.9
41.5	"		58.7
57.2	"	55.4	65.2
30.7	"	43.1	52.8
69.8	"		70.5
57.7	"		65.9
39.1	"	47.7	56.2
29.1	"	43.1	51.3
58.7	"	55.7	65.2
25.4	"	39.5	49.5
83.1	"	64.25	75.1
26.2	"	40.0	48.9
25.2	"	39.7	48.7
79.8	"	62.75	73.75
82.8	"	63.5	75.0
78.65	4.0	68.9	78.3
25.9	5.1	46.4	54.5
68.8	4.1	65.3	74.4
23.9	2.0	44.9	52.6
78.5	4.3	68.5	77.2
23.3	2.0	44.45	51.9
41.35	Air.		58.0
42.2	"		59.4
77.4	"	62.8	73.9

The calculated values of cell volume were then applied to Fig. 1, and, taking account of the theoretical relationships developed by Van Slyke, Wu, and McLean (3), straight lines were drawn to represent the best fitting values of cell volume. These lines were then used for the construction of the scale marked *Vol.* upon Fig. 4.

TABLE IV.

Date.	CO ₂	O ₂	Index of refraction of true plasma.	Remarks.
<i>1925</i>	<i>mm.</i>	<i>mm.</i>		
May 28	29.4	Air.	0.01638	
	42.2	"	0.01678	
May 29	42.2	"	0.01608	
	78.0	"	0.01612	
June 1	39.7	" "	0.01625	
	37.0	22.0	0.01642	
June 4	44.2	Air.	0.01604	Slight hemolysis.
	38.2	3.0	0.01604	
June 5	40.3	Air.	0.01651	
	82.0	"	0.01642	
June 6	38.7	"	0.01581	
	69.0	9.0	0.01601	
June 7	26.0	Air.	0.01593	
	40.5	"	0.01602	
	87.8	"	0.01628	
	39.5	22.5	0.01619	
	39.6	5.3	0.01619	
	85.4	5.5	0.01641	
June 25	27.3	Air.	0.01605	
	40.7	"	0.01611	
	74.5	"	0.01643	
	47.6	24.0	0.01620	Slightest possible trace of hemolysis.
	39.8	5.7	0.01633	Very slight hemolysis.
	82.5	7.0	0.01653	" " "
June 26	79.0	Air.	0.01623	
	65.8	6.0	0.01632	
	40.0	8.4	0.01623	
	37.9	21.2	0.01624	
	40.2	Air.	0.01611	
	21.4	"	0.01608	Slight trace of hemolysis.
Oct. 3	69.9	"	0.01704	
	57.7	"	0.01689	?
	39.05	"	0.01698	
	29.1	"	0.01695	
	58.7	"	0.01716	
Dec. 11	78.65	4.0	0.01634	
	25.9	5.1	0.01661	?
	68.8	4.1	0.01632	
Dec. 14	24.0	4.4	0.01620	

TABLE IV—*Concluded.*

Date.	CO ₂	O ₂	Index of refraction of true plasma.	Remarks.
<i>1928</i>	<i>mm.</i>	<i>mm.</i>		
Dec. 14	27.5	Air.	0.01611	
	83.9	"	0.01632	
	19.0	4.0	0.01615	
	72.2	3.5	0.01634	
	79.15	4.3	0.01634	
	81.5	Air.	0.01642	
	24.1	"	0.01607	
Dec. 17	91.2	"	0.01677	
	25.8	"	0.01642	
	21.1	"	0.01640	
	82.1	6.1	0.01679	Slight hemolysis.
	78.1	4.8	0.01672	" " } Equal.
	22.5	5.8	0.01653	" "

Next these values of *Vol.* and the data for total CO₂ in whole blood and true plasma were used to calculate the concentrations per liter of water of combined carbonic acid in cells and plasma, respectively. From these results values of the Donnan ratio,

$$\frac{[\text{BHCO}_3]_c}{[\text{BHCO}_3]_p}, \frac{[\text{HCO}_3]_c}{[\text{HCO}_3]_p}, \frac{[\text{Cl}]_c}{[\text{Cl}]_p}, \frac{[\text{A}]_c}{[\text{A}]_p}, \frac{[\text{OH}]_c}{[\text{OH}]_p}, \frac{[\text{H}]_c}{[\text{H}]_p}$$

were calculated. Finally, values of *pH_c* were obtained by means of the equation,

$$\text{pH}_c = 6.12 - \log \frac{[\text{H}_2\text{CO}_3]_c}{[\text{BHCO}_3]_c}$$

The method of carrying out the necessary computation is clearly indicated in Van Slyke, Wu, and McLean's paper (3) and will not be repeated here.

The values of *r* and *pH_c* were then fitted as accurately as possible with straight lines on Fig. 1 and these lines were transformed into scales on Fig. 4.

From this nomogram, Fig. 4, it is possible to read directly, or to deduce by simple computation, the magnitudes of all known phenomena of the respiratory mechanism in blood.

The different scales of the nomogram are of different orders of accuracy. Those representing free and combined carbonic acid and combined oxygen are believed to be the best, and are probably quite precise enough for the present requirements of the physiologist. The scale of free oxygen concentration suffers from the fact that in the construction the curves of the Cartesian nomogram have perforce been replaced by straight lines. As a result, this scale is untrustworthy except near the physiological range, say for CO_2 tensions of 44 ± 6 mm. When these four variables alone are in question Fig. 2 is, therefore, to be preferred. These four scales rest upon numerous, consistent, and, as we believe, trustworthy data.

The values of pH, may perhaps be affected with a small and almost precisely constant error. This is, however, a matter of small consequence, at least for most purposes, and the values are probably in all other respects very trustworthy indeed.

The values of *Vol.* and of *r* are more open to question. We have not ourselves been in a position to make a very extensive experimental investigation of these quantities, which, moreover, are not now within the reach of highly accurate measurement. The difficulty is, in part, in the nature of the case. It depends upon the fact that estimation of the variations of *r* rests upon the determination of a quantity which has the character of a second difference, and, similarly, the changes of volume are calculated from the very small changes in the percentage of water in serum. We have, accordingly, been guided in smoothing our data by the theory of Van Slyke, Wu, and McLean (3). No attempt has been made to determine the exact slope, still less the amount of curvature, of the supports of the scales of *Vol.* and of *r*. It may be added that our data seem to suggest that the values of *r* may perhaps be a little greater than those represented on the nomogram, which have been chosen so as to be very nearly in agreement with the equations developed by Van Slyke, Wu, and McLean. Moreover, we cannot now present satisfactory evidence for the identity of *r* in respect to $\bar{\text{Cl}}$ and $\text{H}\bar{\text{C}}\text{O}_2$ distribution. In all other respects our data appear to be consistent with theory.

All these considerations, however, are without much significance for our present purpose, since they concern very small magnitudes or minute differences. They have been pointed

out in order to avoid misunderstandings which might otherwise involve an unduly long, theoretical discussion.

In spite of such uncertainties regarding lesser quantities, it now seems clear that a really considerable change in the nomogram is out of the question. This may be seen by comparing

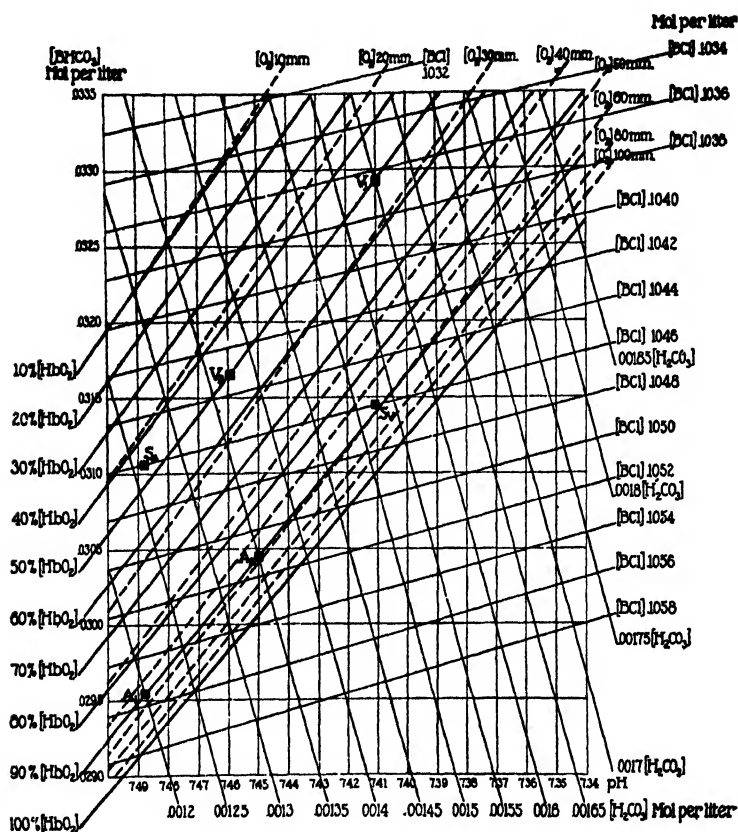


Fig. 5.

Fig. 5, a reproduction of the original nomogram (1), with Fig. 6, an analogous chart constructed by means of a transformation of Fig. 4. The only significant differences between these two figures are those due to the fact that Fig. 6 represents blood which contains a larger quantity of hemoglobin, or to certain differences

in the constants used for calculating the values of the scales in the two cases.

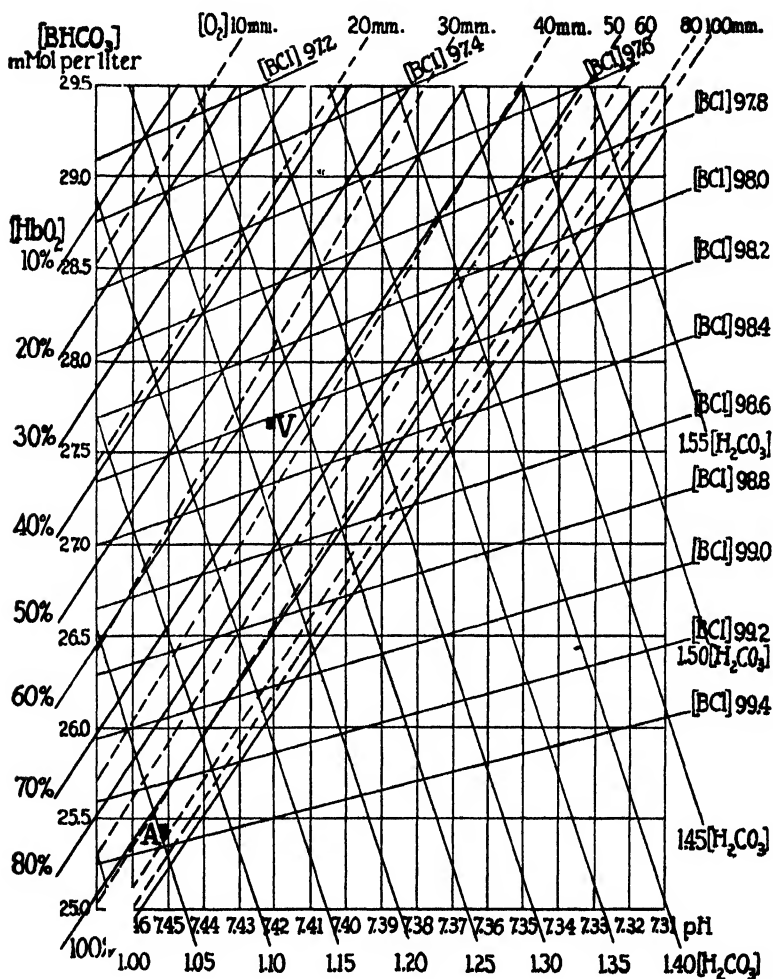


FIG. 6.

It should be noted that the points A_1 , A_2 , V_1 , and V_2 of Fig. 5 were arbitrarily chosen for purposes of illustration and have no real significance.

Fig. 4 is not directly comparable with the nomogram of Van Slyke, Wu, and McLean's paper (3) since the latter was constructed on the model of an earlier nomogram of ours which has proved to be slightly less convenient than Fig. 4. We have, however, transformed Fig. 4 and obtained a result which, except for the differences due to the greater volume of plasma in the human blood, is practically indistinguishable from Van Slyke, Wu, and McLean's chart.

The conclusion seems justified that the *general form* of the nomogram, Fig. 4, represents what may properly be called the law of the blood.

On this chart seven variables are explicitly represented. A large number of others are, however, implicitly defined and may be deduced with the help of very simple calculations. Thus, taking account of the definition of r given by the equation,

$$r = \frac{+[\text{H}]}{+[\text{H}]}$$

we may use the scales of r and of pH_s to obtain values of pH_c . In order to avoid the necessity of making such calculations, it is more convenient to construct the scale of pH_c . This may be done as follows: Choose any value of pH_c , say 7.30, and any convenient pair of values of pH_s , 7.42 and 7.45. Now substitute these values in the equation,

$$\log r = \text{pH}_c - \text{pH}_s$$

obtained from the above equation by taking logarithms and putting $\text{pH} = -\log [\text{H}]$.

$$\begin{aligned}\log r_1 &= 7.30 - 7.42 \\ \log r_2 &= 7.30 - 7.45 \\ r_1 &= 0.76 \\ r_2 &= 0.71\end{aligned}$$

Then the intersection of the lines joining $r = 0.76$ with $\text{pH}_s = 7.42$ and $r = 0.71$ with $\text{pH}_s = 7.45$ is the point corresponding to $\text{pH}_c = 7.30$. In like manner such other points on the pH_c scale as may be necessary to construct the scale are readily found.

This construction has been performed and the result reproduced on the large scale nomogram, Fig. 7, inserted at the end of this paper. Here, too, are represented a number of other variables whose values have been obtained by equally obvious computations and analogous constructions.

It will perhaps suffice to define the variables represented by the several scales of Fig. 7. Taken in order from left to right they are as follows:

I. $(Cl)_s$. The concentration of serum chloride, expressed in millimols per liter of serum.

$$II. r. \text{ Donnan's } r = \frac{[B\bar{H}CO_3]_c}{[B\bar{H}CO_3]_s} = \frac{[\bar{H}CO_3]_c}{[\bar{H}CO_3]_s} = \frac{[Cl]_c}{[Cl]_s} = \frac{[A]_c}{[A]_s} = \frac{[OH]_c}{[OH]_s} = \frac{[H]_c}{[H]_s}$$

Here the brackets represent concentrations per liter of water of serum or cells, as the case may be.

III. $(Cl)_c$. The concentration of cell chloride, expressed as millimols per liter of cells.

IV *a*. $\Delta(Cl)$. The difference, expressed as millimols per liter of blood, compared with arterial blood, in the total amount of chlorides in cells or serum.

IV *b*. Per cent A in cells. The percentage of total blood chloride or bicarbonate present in the cells.

V *a*. *V* of cells. The volume of the cells, expressed as percentage of this volume at about $O_2 = 80$ mm. and $CO_2 = 39$ mm., the point where this volume is 40 per cent of the total blood volume.

V *b*. Per cent H_2O in cells. The percentage of total blood water present in the cells.

VI. BP_c . The base combined with cell protein, expressed as millimols of base per liter of blood.

VII. Cell $B\bar{H}CO_3$. The combined carbonic acid of the cells per liter of blood, expressed: (a) as millimols, and (b) as volumes per cent.

VIII. $(B\bar{H}CO_3)_c$. The combined carbonic acid of the cells per liter of cells, expressed: (a) as millimols per liter, and (b) as volumes per cent.

IX. $(B\bar{H}CO_3)_B$. The combined carbonic acid of whole blood, expressed: (a) as millimols per liter, and (b) as volumes per cent.

X. $(B\bar{H}CO_3)_s$. The combined carbonic acid of serum per liter of serum, expressed: (a) as millimols per liter, and (b) as volumes per cent.

XI. Serum $(B\bar{H}CO_3)$. The combined carbonic acid of serum per liter of blood, expressed: (a) as millimols per liter, and (b) as volumes per cent.

XII. Total CO_2 . The total carbonic acid of blood, expressed: (a) as millimols per liter, and (b) as volumes per cent.

XIII. Δc . The change in concentration of total solute, compared with arterial blood, expressed in millimols per liter of blood.

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XIV. CO_2 . Free carbonic acid, expressed: (a) as millimols per liter of blood, and (b) as millimeters of partial pressure of mercury.

XV a. BP_s . Base bound by protein of serum, expressed as millimols of base per liter of blood.

XV b. pH_s . $-\log \frac{+}{[\text{H}^+]}$ in serum.

XVI. pH_c . $-\log \frac{+}{[\text{H}^+]}$ in cells.

XVII. O_2 . Oxygen tension, expressed as millimeters of partial pressure of mercury.

XVIII. HbO_2 . Combined oxygen, expressed: (a) as millimols per liter of blood, and (b) as per cent of saturation. The scale for total oxygen is so nearly identical with this as to be practically indistinguishable from it.

Great pains have been taken to make these scales consistent with the experimental data, with theory, and with each other. It is hoped that serious errors have been avoided, but in certain cases no great weight is attached to the *absolute* magnitudes of the values.

Across the nomogram two straight lines are drawn. These lines define the equilibria of arterial blood and of venous blood, respectively. The graduated curves tangent to these lines near their point of intersection define the respiratory cycle. They will be discussed below in Section III.

It is important to note, as an aid in constructing similar nomograms from scanty data, that the point of intersection of arterial and venous lines defines the respiratory quotient. The reason for this is easily explained. We first note that the scales of HbO_2 (approximately total oxygen) and total CO_2 are parallel straight lines. Now let any two straight lines cross at any point of a third line parallel to the scales in question. Then the two intersecting lines will enclose portions of the two scales whose ratio is constant; therefore,

$$\text{R.Q.} = \frac{\Delta(\text{total CO}_2)}{\Delta(\text{total O}_2)} = \text{a constant}$$

In the present case the point of intersection is very nearly midway between the two scales. It is evident that this would correspond to a respiratory quotient of approximately 1.0, were it not for the fact that the two scales are spaced in unequal units of length per millimol. As a result of this difference of scale the actual value of the respiratory quotient is found to be approximately 0.82.

On the nomogram broken lines define the position of the points of intersection of arterial and venous lines corresponding to values of the respiratory quotient in the range between 0.70 and 1.00. Each of these lines is the locus of all points correlative with lines of a definite constant slope on a Cartesian nomogram. But on a Cartesian nomogram, having total oxygen and total carbonic acid as coordinates, the slope of a line joining arterial and venous points measures the value of the respiratory quotient.

The nomogram may now be used as a means of discovering the respiratory changes of A.V.B. Tables V, VI, and VII contain the values of the several quantities as read from Fig. 7.

These tables should be compared with the table on page 788 of Van Slyke, Wu, and McLean's paper (3). Once more the differences are due, in the main, to differences in the relative volumes of cells and plasma in the two cases. There are, however, certain slight uncertainties in our data which depend upon the fact that they have been obtained over a period of more than a year during which, no doubt on account of successive bleedings, the hemoglobin content of the blood of A.V.B. was slowly sinking. Thus average values may not be always precisely comparable with each other. The effect of such slight inconsistencies upon the estimation of differences between arterial blood and venous blood is, however, nil.

Table V reveals one fact which merits explicit mention. Reference to the values of BHCO_3 shows that in the passage through the lungs plasma yields 60 per cent and cells only 40 per cent of the total amount of combined carbonic acid eliminated. Now this escape of combined carbonic acid depends upon the reaction, $\text{BHCO}_3 + \text{HP} = \text{BP} + \text{H}_2\text{CO}_3$, and, as the table also shows, cell protein, or essentially hemoglobin, is responsible for 95 per cent of this reaction. Thus it appears that hemoglobin is responsible, under these conditions, for the *transport* of more than 90 per cent of all the carbonic acid excreted. The fact is masked by the accompanying redistribution of chloride ions. Moreover, less than 10 per cent of this 90 per cent is due to buffer action of hemoglobin, and, accordingly, it may be concluded that about 80 per cent of all carbonic acid excreted is dependent for its transport, under normal conditions, upon the oxygen effect upon the acidity of the hemoglobin molecule. In short, hemoglobin

is, though indirectly, hardly less important in the transport of carbonic acid than in that of oxygen itself. This subject has been fully discussed in an earlier paper (6); but the present estimates are much more exact.

Fig. 7 may be used to define any other conditions of equilibrium within the common ranges of arterial and mixed venous blood. To this end it will be found a very powerful instrument. Indeed an alignment chart is probably the only means of pre-

TABLE VI.
Serum.

		Arterial.	Venous.	Δ
H ₂ O	cc. per l. serum.....	915.4	914.3	-1.0
B	mm " " "	140.1	141.1	+1.0
Cl	" " " "	99.37	98.22	-1.15
BP	" " " "	15.34	15.27	-0.07
BHCO ₃	" " " "	25.40	27.66	+2.26
H ₂ CO ₃	" " " "	1.17	1.38	+0.21
Total CO ₂	" " " "	26.58	29.04	+2.46

TABLE VII.
Cells.

		Arterial.	Venous.	Δ
H ₂ O	cc. per l. cells.....	649.5	654.2	+5
B	mm " " "	120.7	119.3	-1.4
Cl	" " " "	50.98	53.21	+2.23
BP	" " " "	56.70	51.18	-5.52
BHCO ₃	" " " "	12.99	14.96	+1.97
H ₂ CO ₃	" " " "	0.85	0.99	+0.14
Total CO ₂	" " " "	13.86	15.95	+2.09
Combined O ₂	" " " "	21.2	14.3	-6.9

senting such a great mass of quantitative information in compact form. But it must not be forgotten that most of the scales represent mathematical functions of a quite secondary character and that seven scales suffice completely to define the system of the blood in accordance with our present knowledge. Given these seven scales, all the others may be deduced without the use of any information beyond that involved in the construction of these fundamental scales. From a logical standpoint Fig. 4

is one among several possible complete expressions of the nature of blood as a physicochemical system, in accordance with present knowledge. We believe that it contains neither more nor less than the necessary and sufficient number of scales, although, within limits, a different choice of variables is open.

II.

The synthesis of our knowledge of the equilibria of the blood for the narrow physiological range of variations is completely represented by Fig. 4. We may now proceed to a systematic exposition of the different partial aspects of the phenomenon.

It has long been customary to represent two of these, the oxygen dissociation at different tensions of carbonic acid and the carbonic acid dissociation at different oxygen saturations, with the help of contour line charts. A third aspect is defined by the familiar equation,

$$\frac{+}{[\text{II}]} = k' \frac{[\text{H}_2\text{CO}_3]}{[\text{BHCO}_3]}$$

which is the analytical expression of a similar Cartesian nomogram.

The possibility of thus dealing with three variables at a time is nothing but the expression of the fact that, aside from variations in O_2 and CO_2 tensions, the blood is assumed to be in a steady state, although in certain special cases this restriction is unnecessary. The fact that it is possible thus to define the variations of any three variables, independently of the other four, may readily be demonstrated as follows: Choose any three scales, say u , v , and w , on Fig. 4. Then, if values of any two of the three variables are given, *e.g.*, u_1 , v_1 , or u_2 , w_2 , or v_3 , w_3 , the third is determined. This is true because two points determine a straight line and the intersection of this line with the scale of the unknown variable determines the value, w_1 or v_2 or u_3 , of this variable.

Now among 7 variables, taking 3 at a time, there are 35 combinations. Accordingly, the three cases above mentioned are but 3 among 35 cases necessary for an exhaustive description. Moreover, in each of these 35 cases 3 variables are involved. Accordingly, it is possible, in each case, to construct three contour line charts, taking in turn u and v , u and w , and v and w as the correlatives of x and y , the Cartesian coordinates. Thus

a complete treatment involves the construction of 105 Cartesian contour line charts.

These 105 charts fall into 21 sets of 5 each. There are, in fact, 21 combinations, taking 2 at a time, among 7 variables. Therefore, there are 21 pairs of Cartesian coordinates. When 2 of the variables have been chosen as Cartesian coordinates, 5 remain. Accordingly, they yield 5 families of contour lines. Evidently the 5 members of each of these 21 sets of contour line charts form by superposition a Cartesian nomogram, which is the complete expression of the equilibrium and the equivalent in all respects of Fig. 4. Figs. 8 to 112 present these 105 charts arranged according to the plan of Table VIII.

The construction of any one of these charts may be explained as follows: Let the variables in question be x , y , and z , represented by the scales u , v , and w , of Fig. 4, and let it be required to draw contour lines representing values of z on a Cartesian background of x and y . Choose suitable values for the z contour lines and find on w the points corresponding to these values. Through each of these points pass several straight lines and read the pairs of values of x and y defined by the intercepts of these lines on u and v . After tabulating the data thus obtained, it only remains to transfer them to a Cartesian background of x and y , and to join each set of points corresponding to each of the values of z .

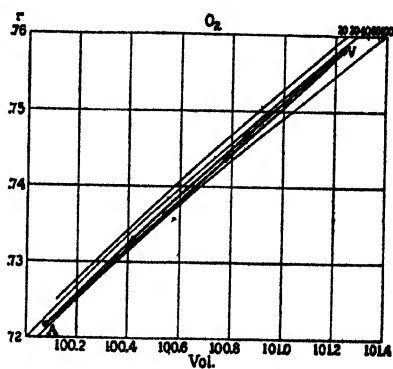
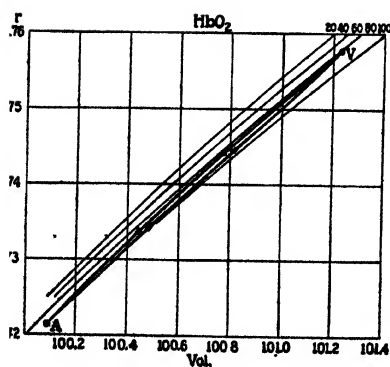
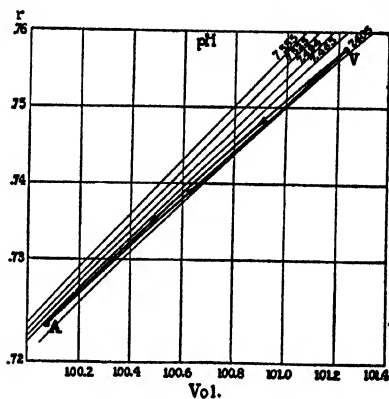
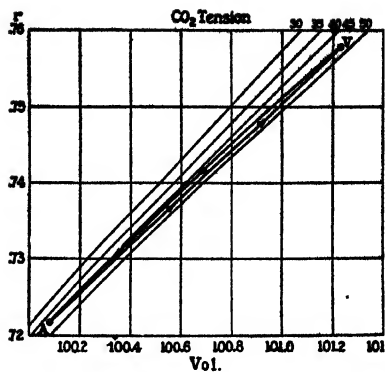
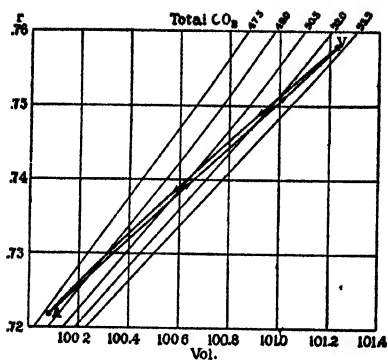
On each of Figs. 8 to 112 the respiratory cycle and the arterial and venous points are represented. The cycle has been calculated from the estimates of Section III of this paper.

The figures contain nothing that is not also contained in Fig. 4. But, like the two familiar charts, Figs. 66 and 111, which they include, they facilitate the understanding of the details of the respiratory process and systematically represent in turn all these details.

In a few instances the choice of variables renders this representation insufficiently explicit. Thus the oxygen dissociation curves for constant hydrogen ion concentration of the corpuscles are slightly different from those for constant hydrogen ion concentration of the plasma. They may indeed be obtained very simply with the help of values of r . But the result is not particularly easy to foresee. We shall return to this question in a later paper.

TABLE VIII.

Page.	Figs.	Cartesian coordinates.	Contour lines.
403	8-12	r, v	Total CO_2 , CO_2 tension, pH , HbO_2 , O_2 tension
404	13-17	r , total CO_2	v , CO_2 tension, pH , HbO_2 , O_2 tension
405	18-22	r , CO_2 tension	v , total CO_2 , pH , HbO_2 , O_2 tension
406	23-27	r , pH	v , total CO_2 , CO_2 tension, HbO_2 , O_2 tension
407	28-32	r , HbO_2	v , total CO_2 , CO_2 tension, pH , O_2 tension
408	33-37	r , O_2 tension	v , total CO_2 , CO_2 tension, pH , HbO_2
409	38-42	r , total CO_2	r , CO_2 tension, pH , HbO_2 , O_2 tension
410	43-47	v , CO_2 , O_2 tension	r , total CO_2 , pH , HbO_2 , O_2 tension
411	48-52	v , pH	r , total CO_2 , CO_2 tension, HbO_2 , O_2 tension
412	53-57	v , HbO_2	r , total CO_2 , CO_2 tension, pH , O_2 tension
413	58-62	v , O_2 tension	r , total CO_2 , CO_2 tension, pH , HbO_2
414	63-67	Total CO_2 , CO_2 tension	r , v , pH , HbO_2 , O_2 tension
415	68-72	Total CO_2 , pH	r , v , CO_2 tension, HbO_2 , O_2 tension
416	73-77	Total CO_2 , HbO_2	r , v , CO_2 tension, pH , O_2 tension
417	78-82	Total CO_2 , O_2 tension	r , v , CO_2 tension, pH , HbO_2
418	83-87	CO_2 tension, pH	r , v , total CO_2 , HbO_2 , O_2 tension
419	88-92	CO_2 tension, HbO_2	r , v , total CO_2 , pH , O_2 tension
420	93-97	CO_2 tension, O_2 tension	r , v , total CO_2 , pH , HbO_2
421	98-102	pH , HbO_2	r , v , total CO_2 , CO_2 tension, O_2 tension
422	103-107	pH , O_2 tension	r , v , total CO_2 , CO_2 tension, HbO_2
423	108-112	HbO_2 , O_2 tension	r , v , total CO_2 , CO_2 tension, pH



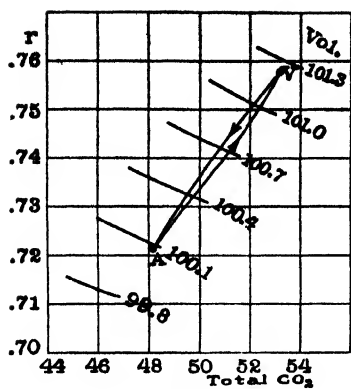


FIG. 13.

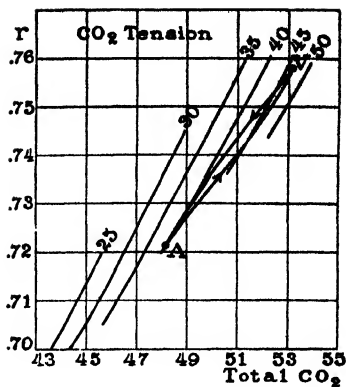


FIG. 14.

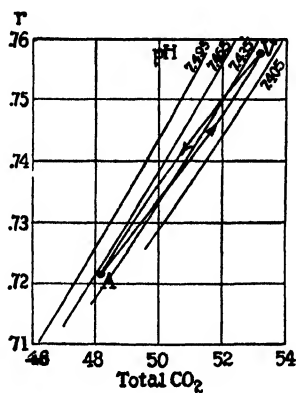


FIG. 15.

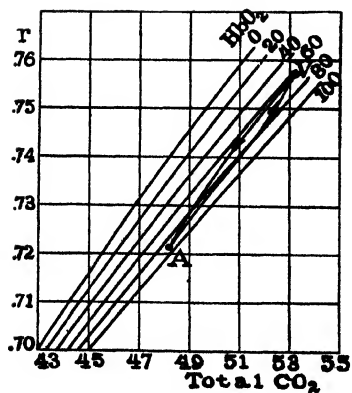


FIG. 16.

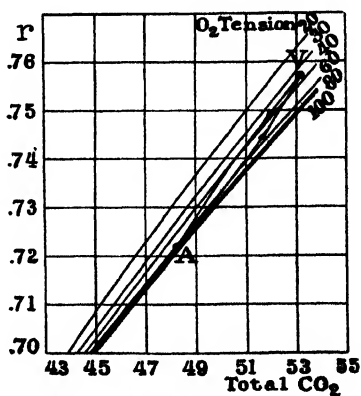


FIG. 17.

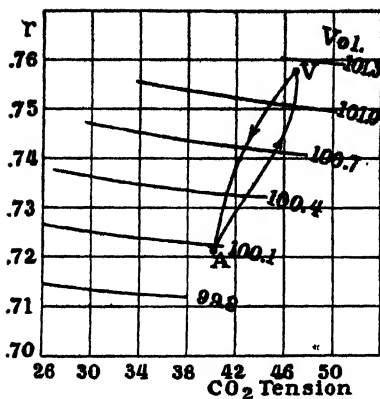


FIG. 18.

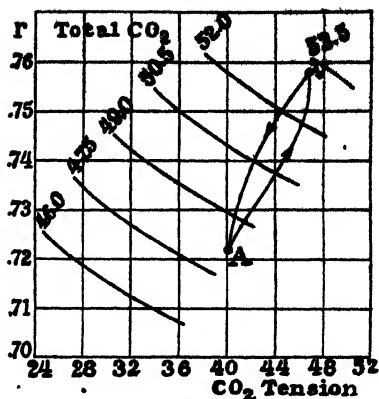


FIG. 19.

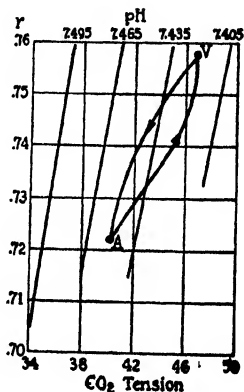


FIG. 20.

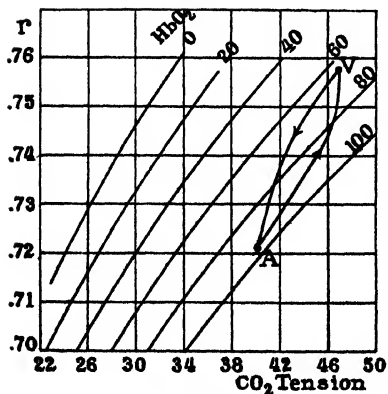


FIG. 21.

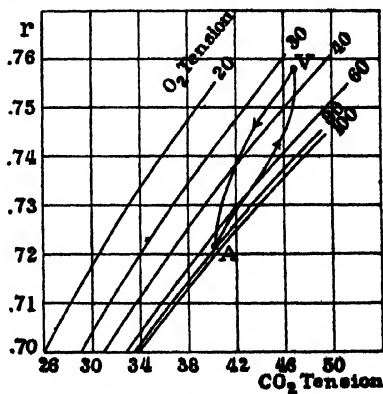


FIG. 22.

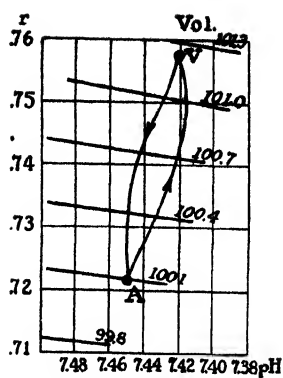


FIG. 23.

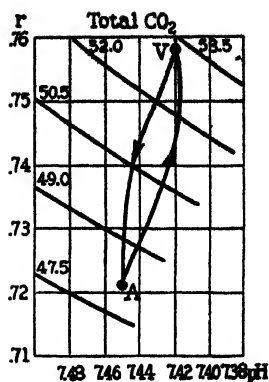


FIG. 24.

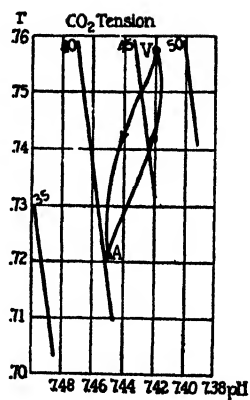


FIG. 25.

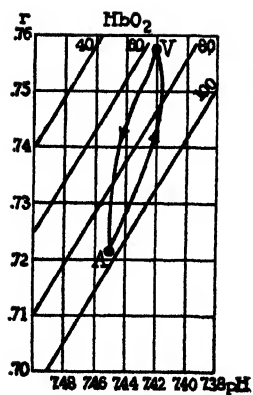


FIG. 26.

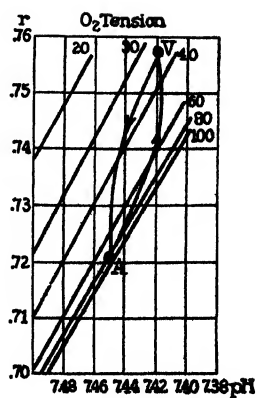
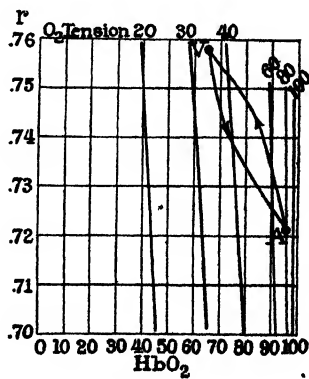
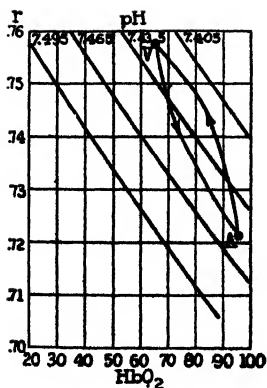
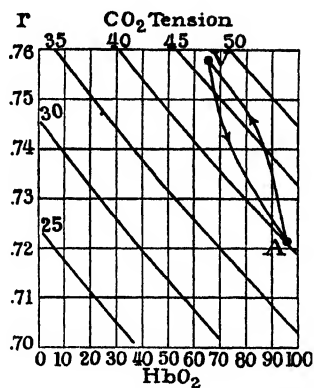
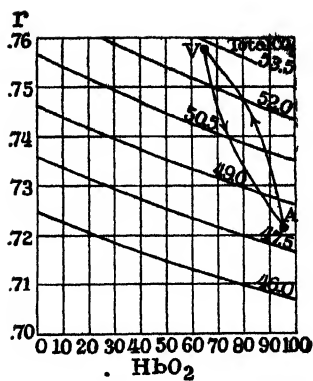
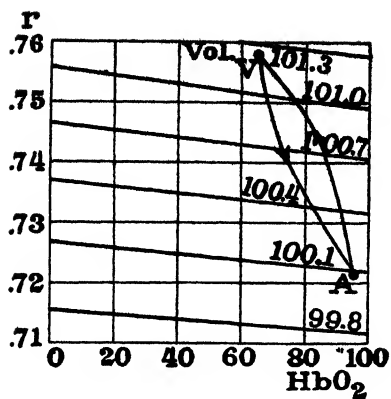


FIG. 27.



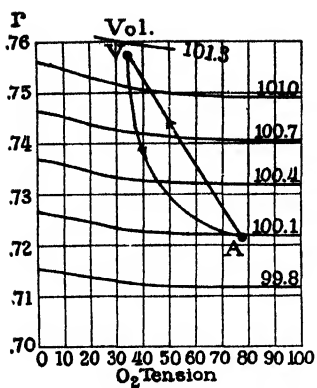


FIG. 33.

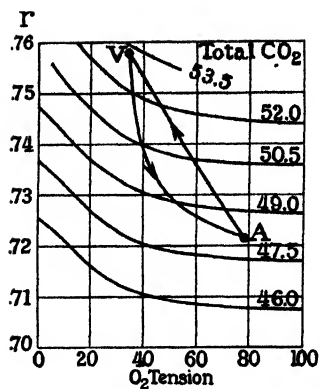


FIG. 34.

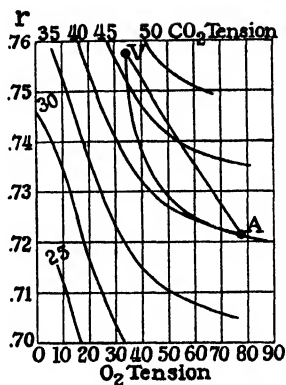


FIG. 35.

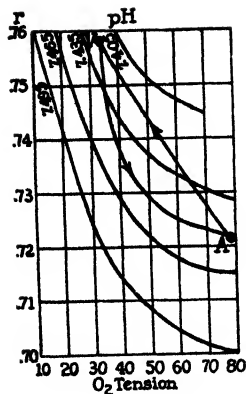


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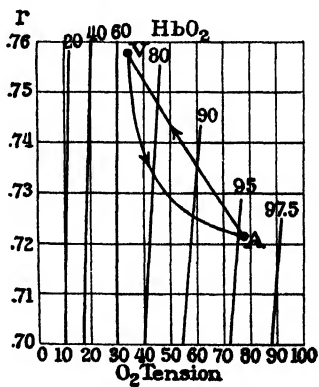


FIG. 37.

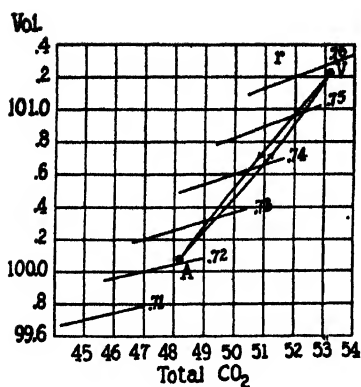


FIG. 38.

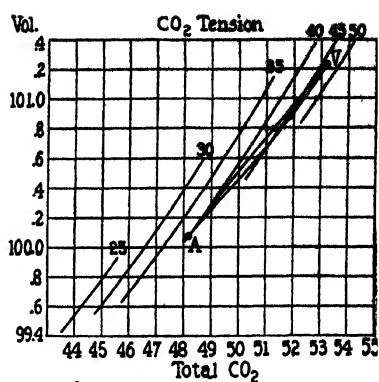


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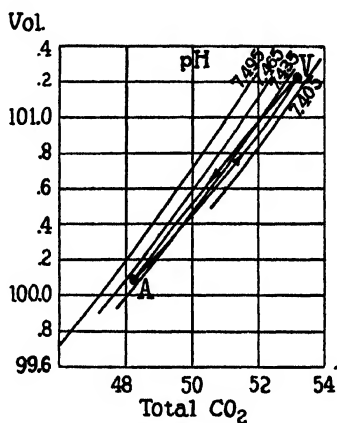


FIG. 40.

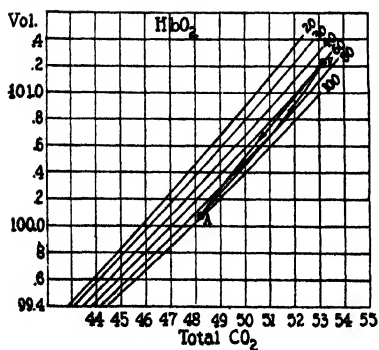


FIG. 41.

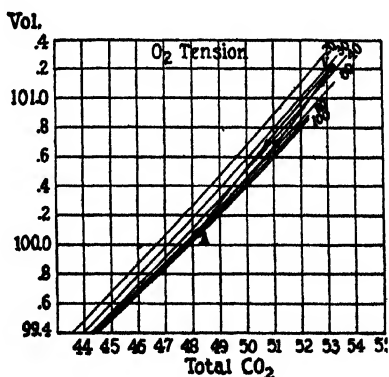
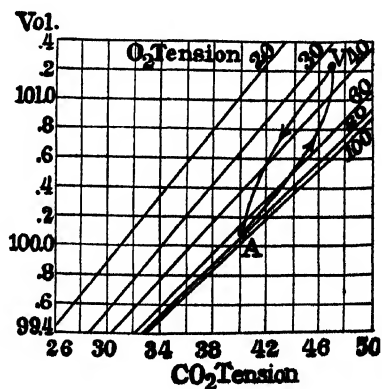
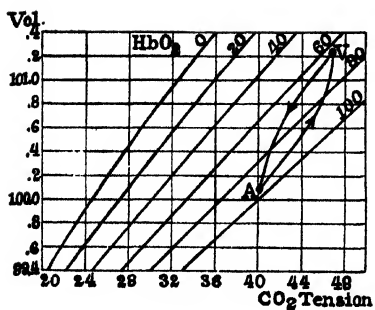
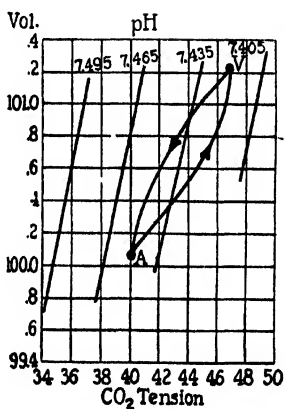
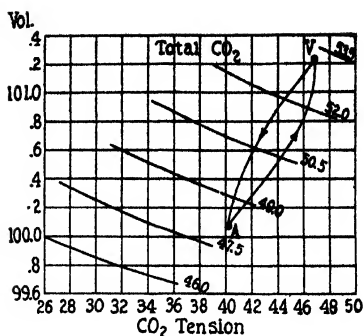
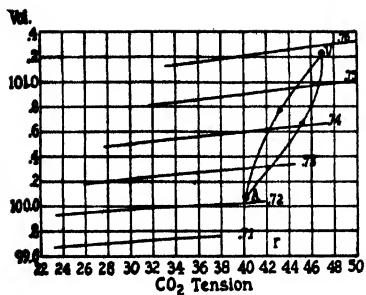


FIG. 42.



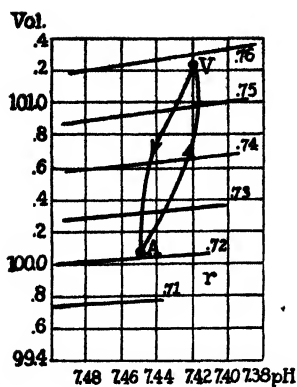


FIG. 48.

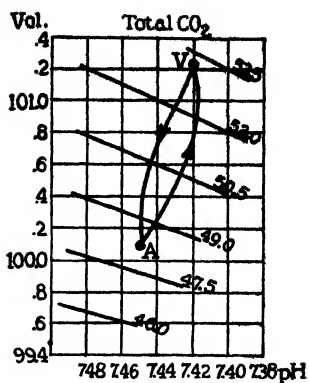


FIG. 49.

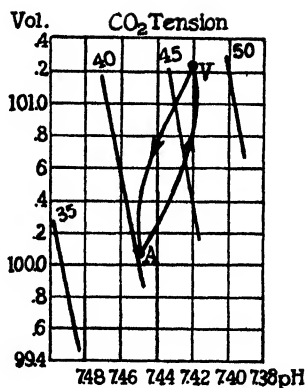


FIG. 50.

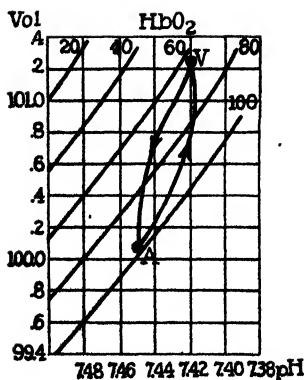


FIG. 51.

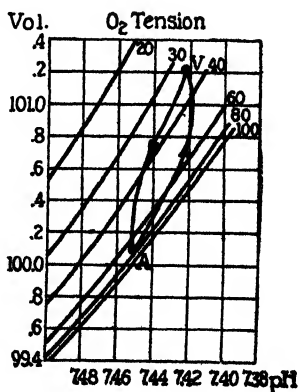


FIG. 52.

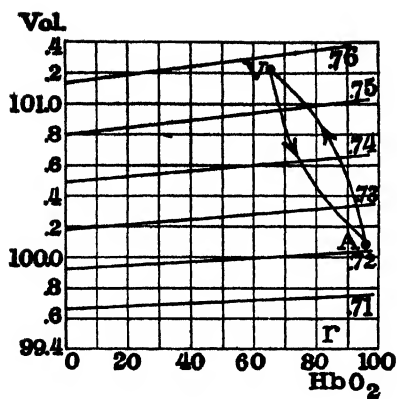


FIG. 53.

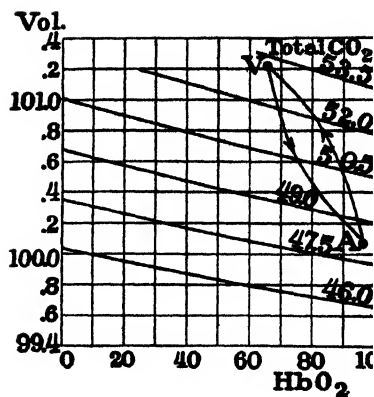


FIG. 54.

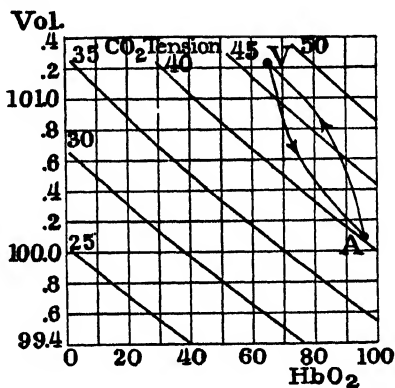


FIG. 55.

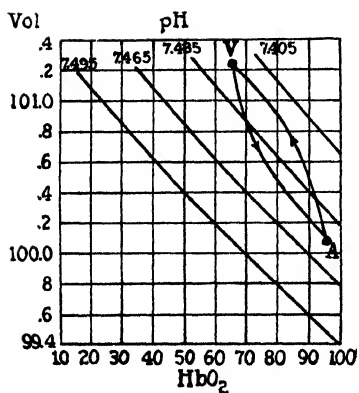


FIG. 56.

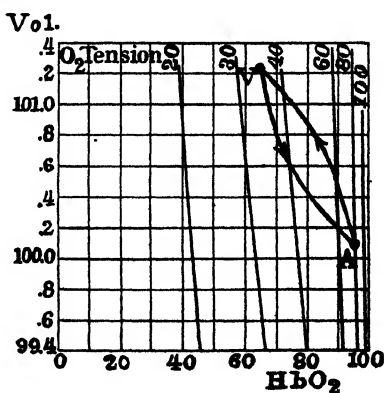


FIG. 57.

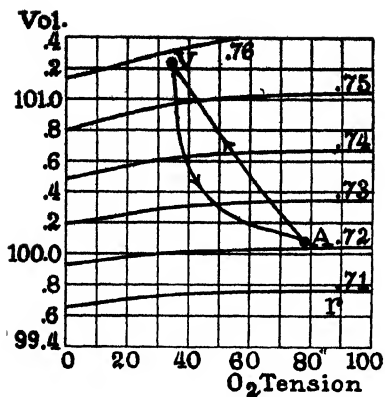


FIG. 58.

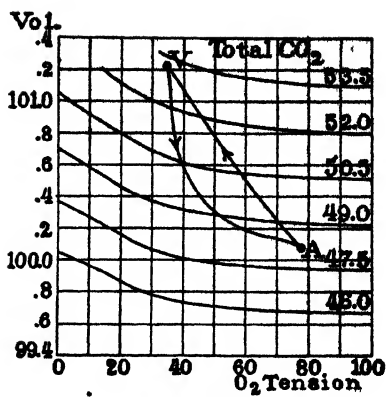


FIG. 59.

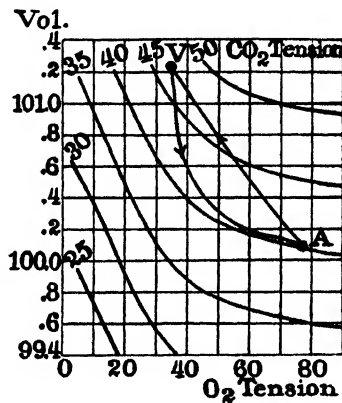


FIG. 60.

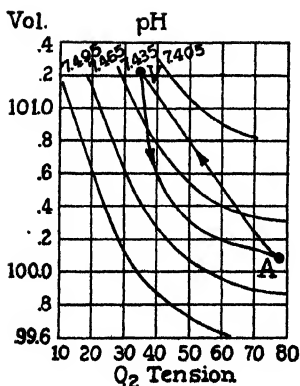


FIG. 61.

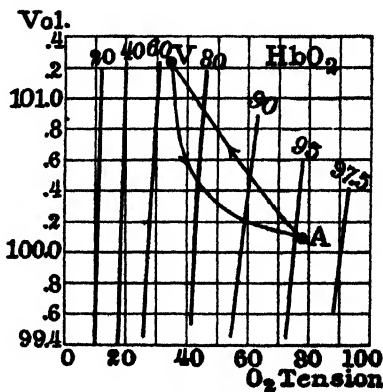


FIG. 62.

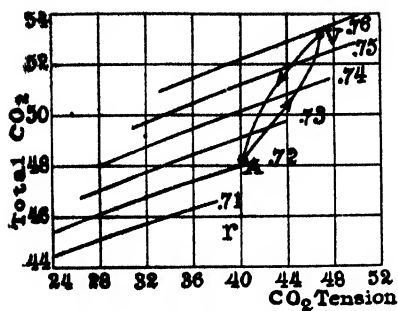


FIG. 63.

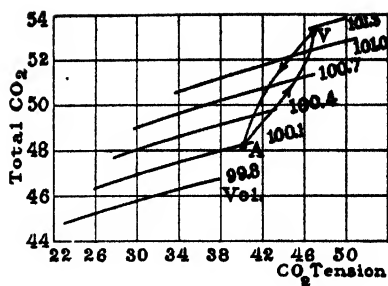


FIG. 64.

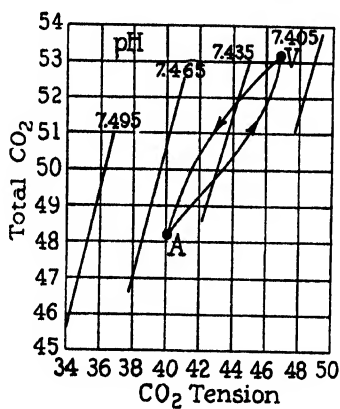


FIG. 65.

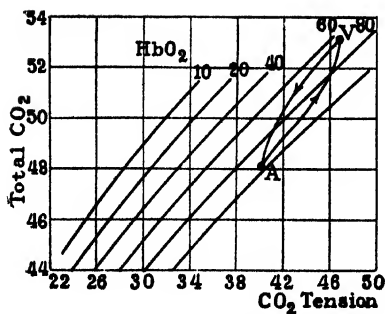


FIG. 66.

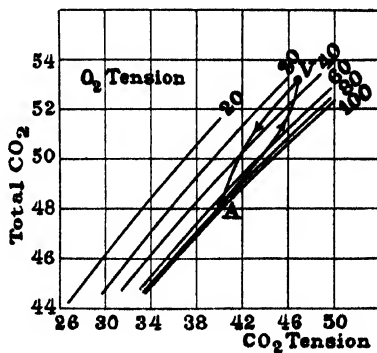


FIG. 67.

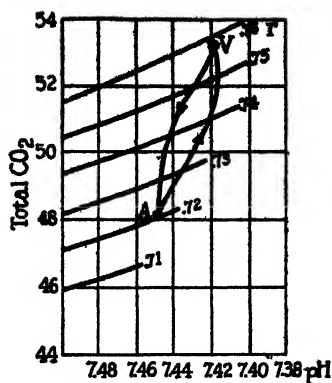


FIG. 68.

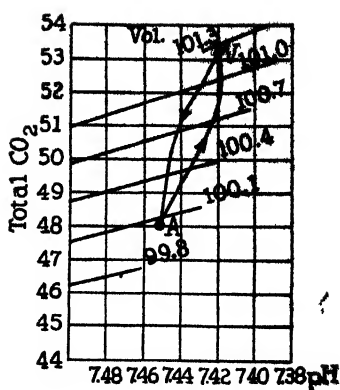


FIG. 69.

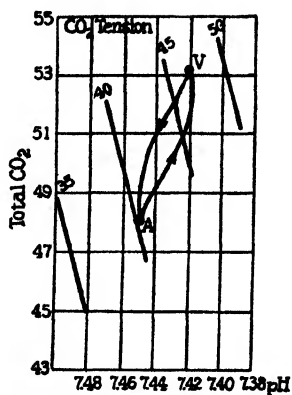


FIG. 70.

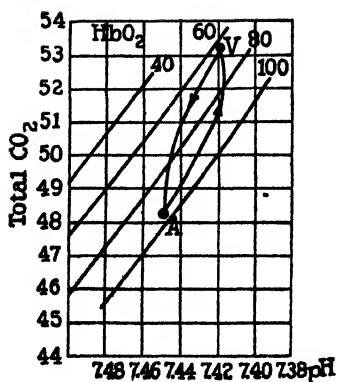


FIG. 71.

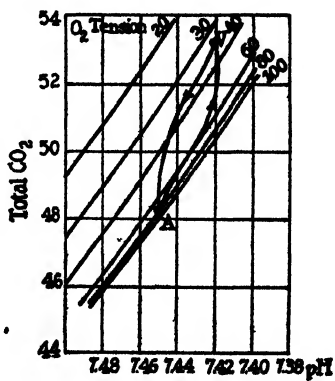


FIG. 72.

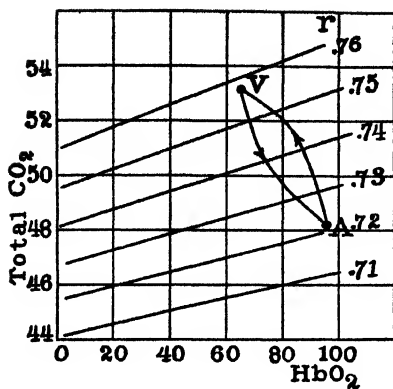


FIG. 73.

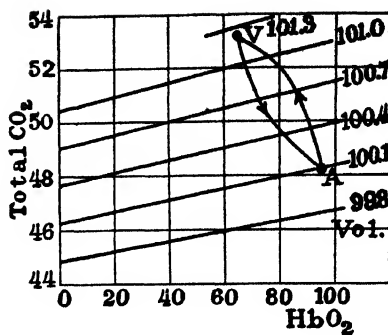


FIG. 74.

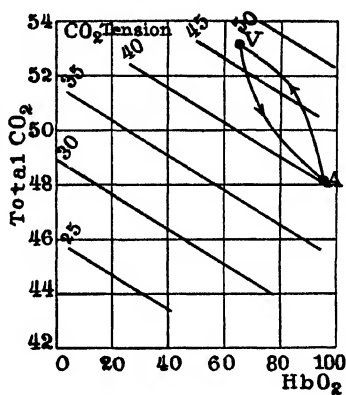


FIG. 75.

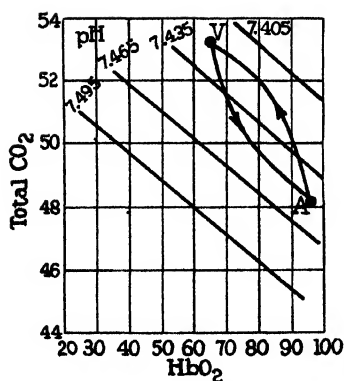


FIG. 76.

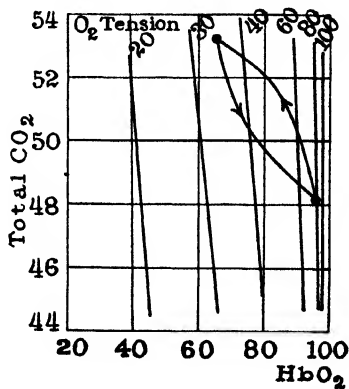
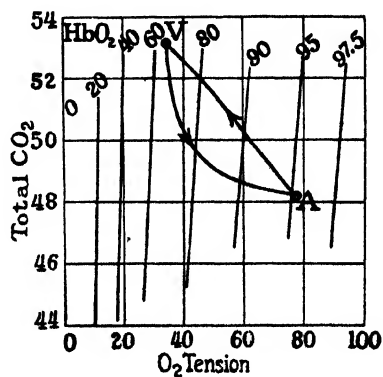
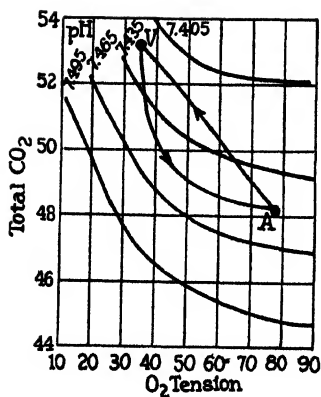
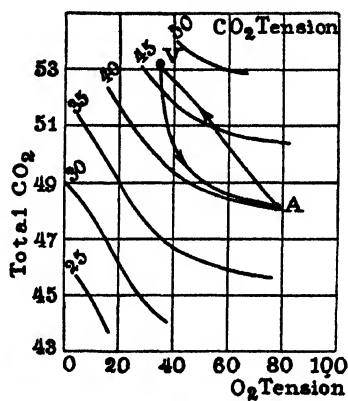
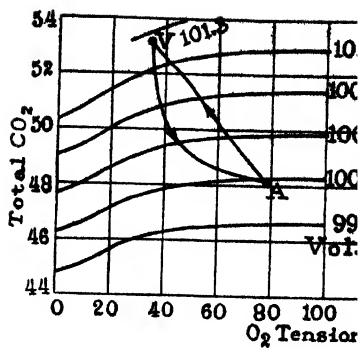
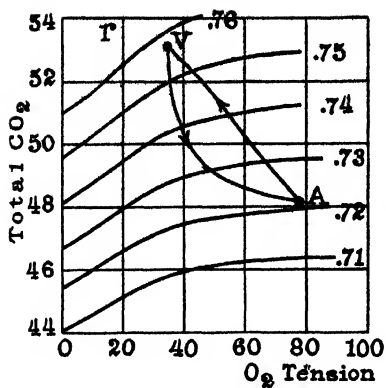


FIG. 77.



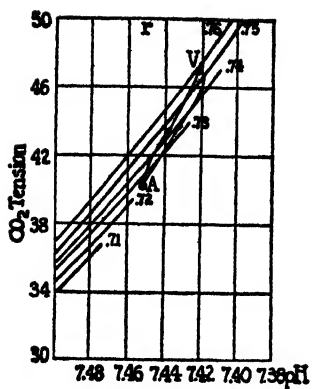


FIG. 83.

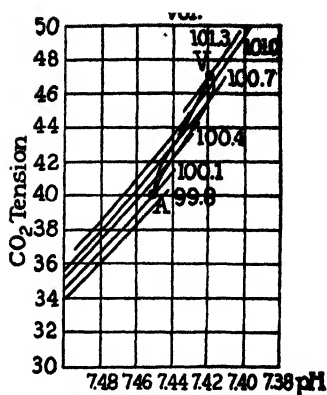


FIG. 84.

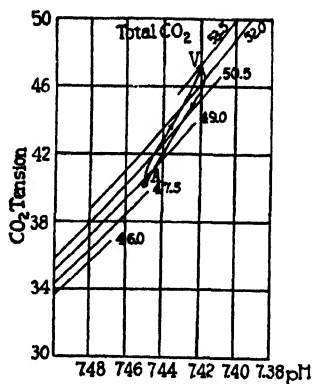


FIG. 85.

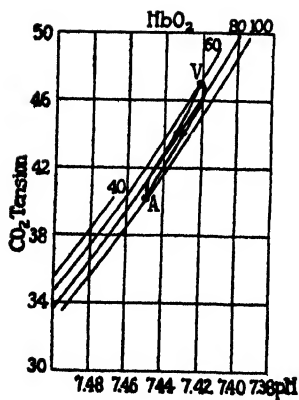


FIG. 86.

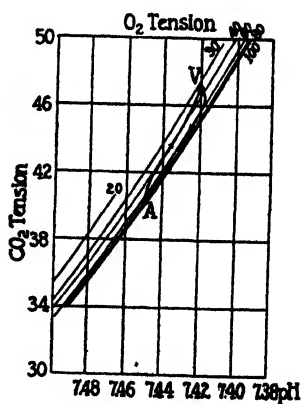


FIG. 87.

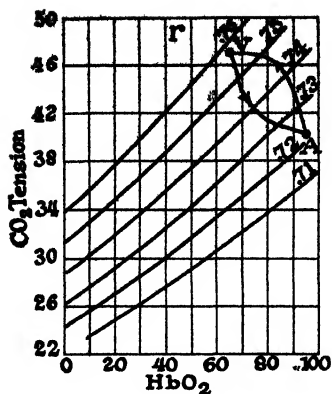


FIG. 88.

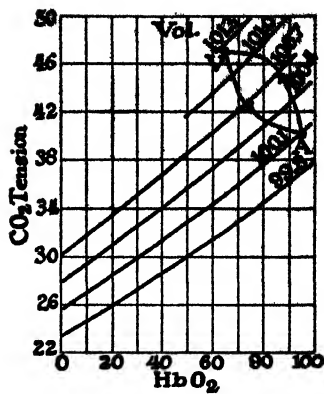


FIG. 89.

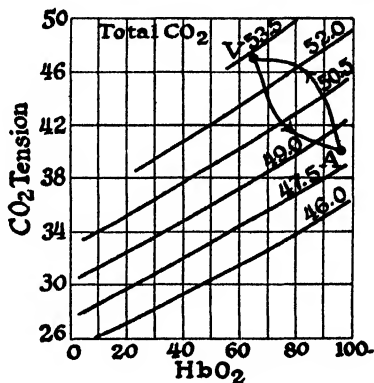


FIG. 90.

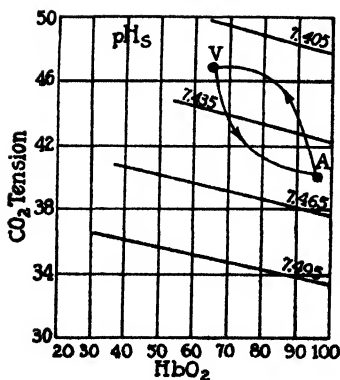


FIG. 91.

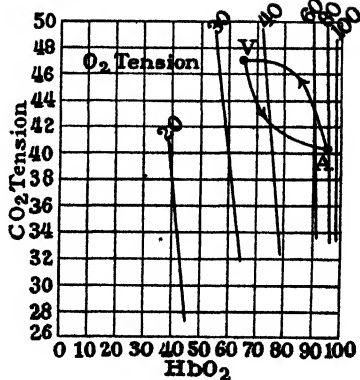


FIG. 92.

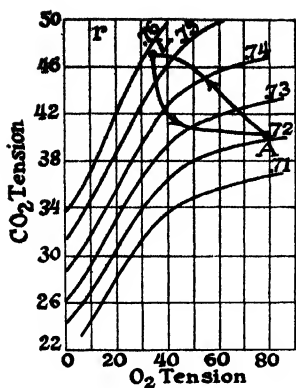


FIG. 93.

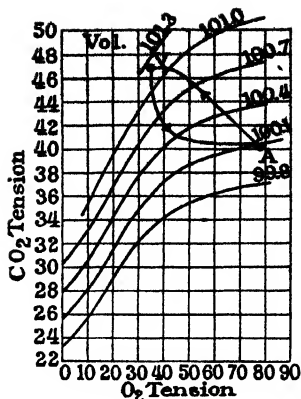


FIG. 94.

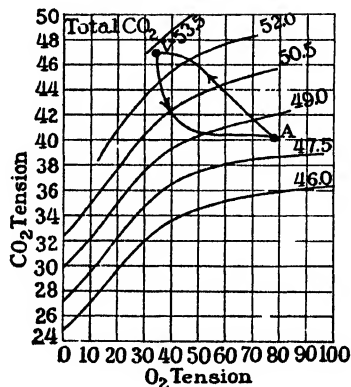


FIG. 95.

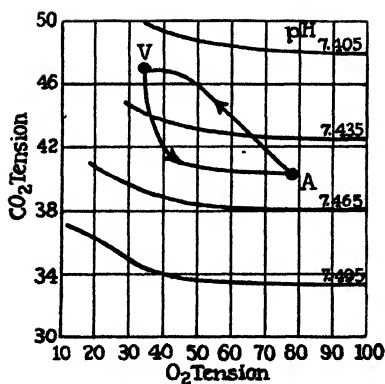


FIG. 96.

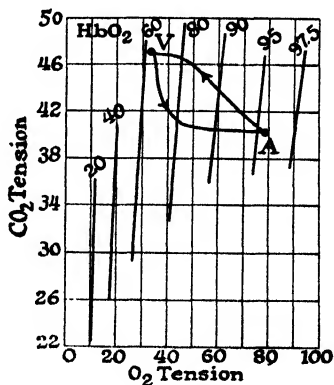


FIG. 97.

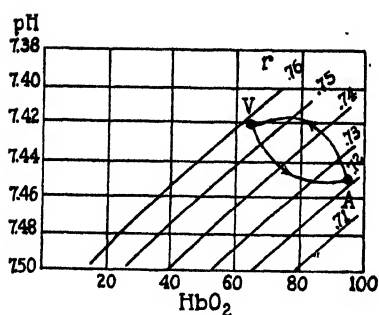


FIG. 98.

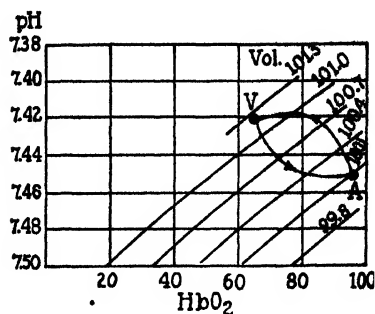


FIG. 99.

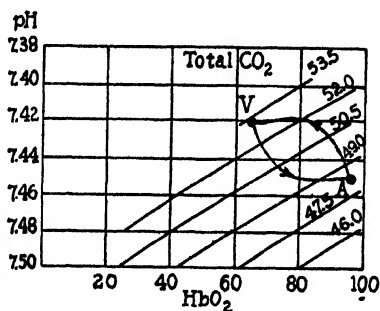


FIG. 100.

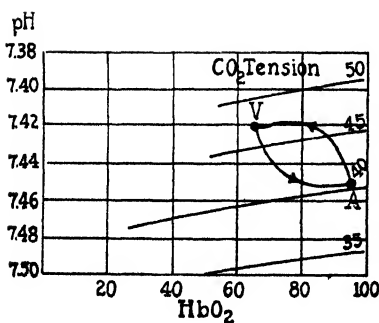


FIG. 101.

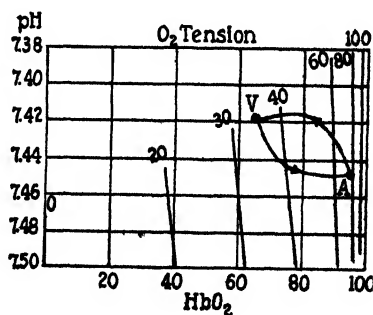


FIG. 102

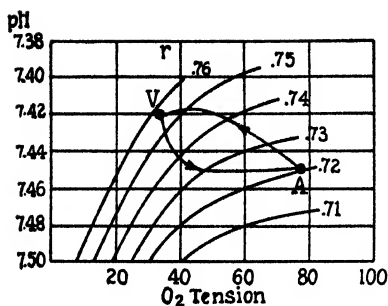


FIG. 103.

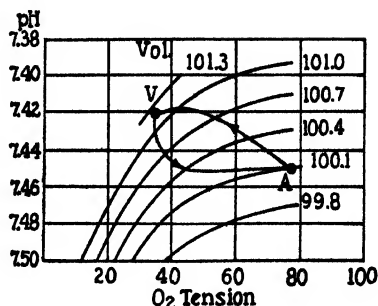


FIG. 104.

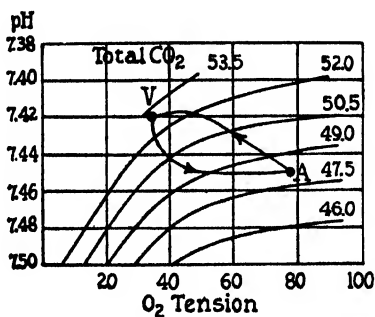


FIG. 105.

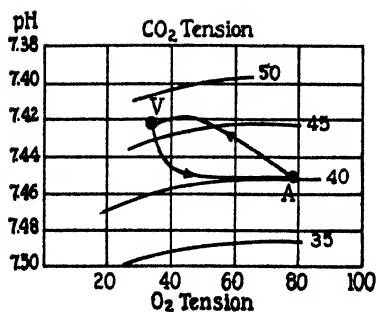


FIG. 106.

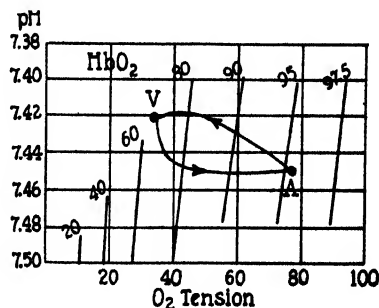


FIG. 107.

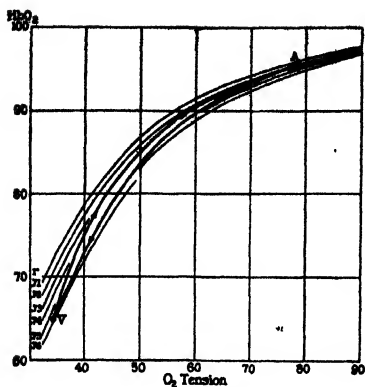


FIG. 108.

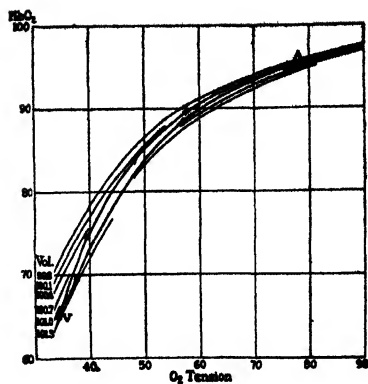


FIG. 109.

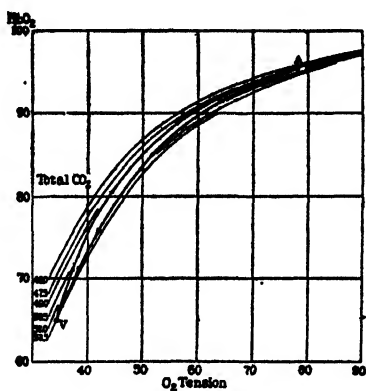


FIG. 110.

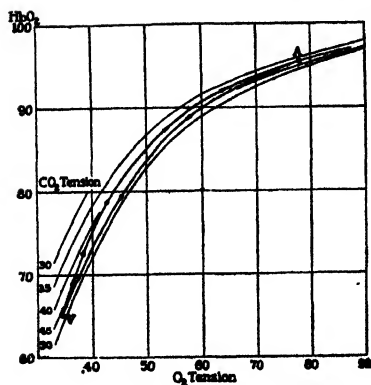


FIG. 111.

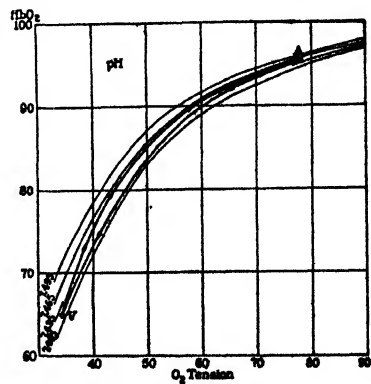


FIG. 112.

III.

The results of the above inquiry may now be used in an investigation of the implications of the diffusion theory concerning the process by which venous blood becomes arterial or arterial blood, venous.

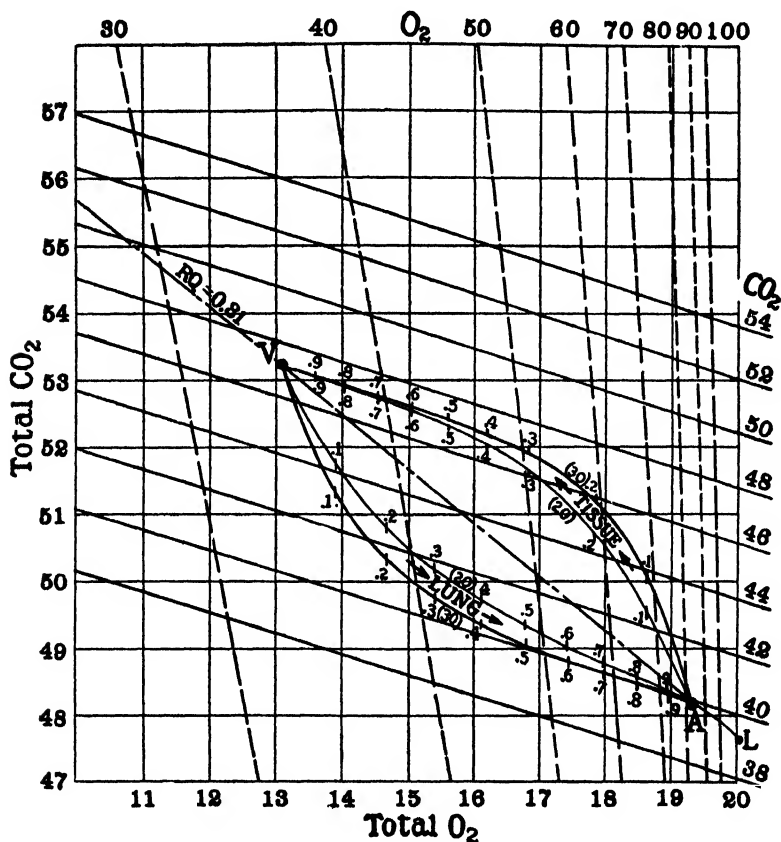


FIG. 113.

In Fig. 113 the abscissæ are values of total oxygen, the ordinates, values of total CO₂, while values of oxygen tension and CO₂ tension appear as contour lines. Fundamentally, therefore, the figure is a large scale drawing of a portion of Fig. 2. Three points are marked on the figure: *L*, corresponding to the O₂

and CO_2 tensions of alveolar air, as directly determined; A , the arterial blood point, determined by repeated analyses of blood drawn from the radial artery; and V , the venous blood point. The position of this point is less accurately known. It must, however, fall somewhere on the line marked $RQ = 0.81$, since this was the value of A.V.B.'s respiratory quotient for the conditions now under consideration, and it is evident that on this diagram any straight line drawn through the arterial point is a respiratory quotient line. The slope of the line is the measure of the respiratory quotient. Therefore, it is necessary to know only one fact regarding the mixed venous blood in order to determine the position of the venous point on the diagram. We have estimated the CO_2 tension of this blood as 47 mm. and have placed the point accordingly.

This procedure has obvious applications, which we are now investigating, to the estimation of the blood flow.

With the Cartesian coordinates, the O_2 and CO_2 contour lines, and the points L , A , and V , once established, it is possible to take another step.

The rate of increase of the oxygen content of an infinitesimal portion of a capillary column of blood in the lungs must be proportional to the difference between the alveolar oxygen tension, $[\text{O}_2]_L$, and that of the blood, $[\text{O}_2]_p$:

$$-\frac{d(\text{total O}_2)}{dt} = a_1([\text{O}_2]_L - [\text{O}_2]_p) = a_1\Delta\text{O}_2$$

Similarly,
$$\frac{d(\text{total CO}_2)}{dt} = a_2([\text{CO}_2]_L - [\text{CO}_2]_p) = a_2\Delta\text{CO}_2$$

Dividing,
$$\frac{d(\text{total CO}_2)}{d(\text{total O}_2)} = \frac{a_2}{a_1} \times \frac{\Delta\text{CO}_2}{\Delta\text{O}_2}$$

The value of the constant term $\frac{a_2}{a_1}$ is not accurately known. For water its value is about 20, for the tissues about 30. But we are here concerned with conditions which are hard to define, since, to mention only one complication, the amount of mixing within the capillary, and hence the extent to which the exchanges between red cells and plasma are adjusted, remain unknown. We

shall therefore make no attempt to estimate the value of $\frac{a_2}{a_1}$, but shall employ in turn the round values of 20 and 30 in order to discover, if possible, the general characteristics of the diffusion process.

For Fig. 113

$$\frac{d(\text{total CO}_2)}{d(\text{total O}_2)} = \frac{dy}{dx}$$

Therefore, it is evident that all points such that

$$\frac{a_2}{a_1} \times \frac{\Delta \text{CO}_2}{\Delta \text{O}_2} = m = \text{a negative constant}$$

or in words such that the difference between the CO_2 tension at the point and the CO_2 tension of the alveolar air, divided by the difference between the O_2 tension at the point and the O_2 tension of alveolar air is constant, are points which define a slope on the Cartesian coordinates.

The meaning of this slope may be easily understood from the following considerations. Instead of speaking of a point on the chart as defining a given condition of the blood, we may speak of the blood as existing at a point on the chart. Then, in order to reach the arterial point the blood may be said to describe a curve upon the chart. Now the direction in which the blood must be moving when at the point p is that of the slope, m_p , in question. In other words, this slope is the slope of the tangent, at the point p , to the curve over which the blood must pass as a result of a diffusion process. This is true because, as already explained, when the blood is at the point p ,

$$\frac{dy}{dx} = \frac{d(\text{total CO}_2)}{d(\text{total O}_2)} = \frac{a_2}{a_1} \times \frac{([\text{CO}_2]_L - [\text{CO}_2]_p)}{([\text{O}_2]_L - [\text{O}_2]_p)} = \frac{a_2}{a_1} \times \frac{(\Delta \text{CO}_2)_p}{(\Delta \text{O}_2)_p} = m_p$$

It is, accordingly, convenient to draw a family of contour lines, each one such that for every point of the contour line

$$\frac{a_2}{a_1} \times \frac{([\text{CO}_2]_L - [\text{CO}_2]_p)}{([\text{O}_2]_L - [\text{O}_2]_p)} = \frac{a_2}{a_1} \times \frac{(\Delta \text{CO}_2)_p}{(\Delta \text{O}_2)_p} = m_p = \text{a constant}$$

Here $[\text{CO}_2]_p$ and $[\text{O}_2]_p$ are the CO_2 tension and O_2 tension corresponding to any point, p , of the contour line and $[\text{CO}_2]_L$ and $[\text{O}_2]_L$ the CO_2 tension and the O_2 tension corresponding to the alveolar air point, L .

Next, taking $\frac{a_2}{a_1} = 20$, the characteristic slope, $\frac{dy}{dx} = \frac{d(\text{total CO}_2)}{d(\text{total O}_2)}$, defined by each contour line, is calculated, and a large number of short parallel lines of the calculated slope, each intersecting the contour line, are drawn. It now remains to join the point V and the point L by means of a curve which cuts each of these contour lines so that the tangent to the curve at each point of intersection with a contour line is parallel with the characteristic slope defined by the contour line. The curve thus constructed is the required representation of the diffusion process in the lung.

Taking $\frac{a_2}{a_1} = 30$, a similar curve is obtained. These two curves are represented on Fig. 113 and are marked "LUNG (20)" and "LUNG (30)."

The analogous curves for the tissue diffusion process, assuming a condition in which the local venous blood is of the same composition as the mixed venous blood, are somewhat more difficult to obtain and also more uncertain. This depends upon the fact that, in the absence of information concerning O_2 and CO_2 tensions within the tissues, it is necessary to proceed by a method of successive approximations. Thus have been obtained the curves marked "TISSUE (20)" and "TISSUE (30)" on Fig. 113.

The researches of Krogh (13) justify the belief that the outer curves "LUNG (30)" and "TISSUE (30)" more nearly represent the process as it might take place under ideal conditions. But those peculiarities of the blood which are responsible for the wide separation of the diffusion curve for the lung from that for the tissue are in part dependent upon heterogeneous reactions between cells and plasma. Therefore, taking account of the uncertainty regarding the completeness of such reactions during the passage of blood through capillaries, we shall employ the curves (20). In so doing we wish merely to imply that the differences between the diffusion process in the lung and the reverse process in the tissues are probably *at least* as great as

these two curves indicate. In any event, it is evident that the cycle marked (20) and that marked (30) are not very unlike. They are, in fact, necessarily very much alike in all but magnitude; *i.e.*, in radius of curvature. Concerning the effect of inadequate mixing more fundamentally to modify the process, we have no information.

Another similar graphical integration makes possible the graduation of the two cycles so as to represent time. No doubt the results are rough approximations; even so they can hardly be meaningless. We have expressed these results by dividing the time of each process into tenths and marking these divisions on the two cycles. They may be compared with the results obtained by Bohr (14) at a time when knowledge of the process was less complete.

On each of Figs. 8 to 112 the cycle (20) has been represented. On the large alignment chart, Fig. 7, two curves are drawn. These are the envelopes of all lines corresponding to points on the cycle (20). Every tangent to these curves represents some point on cycle (20) of Fig. 113. The time scales have also been placed on these envelopes. Thus a tangent at point 0.5 on the lung envelope roughly represents the condition of an infinitesimal portion of a capillary column of blood, when half way through a lung capillary of average dimensions and relations.

Finally, it is interesting to consider the variations in each variable separately, during the cycle. These are represented for the seven fundamental variables and also, because of anomalous fluctuations, for pH_c , on Figs. 114 to 121. In each case time is taken as abscissa and the time of passage through the capillary divided into tenths.

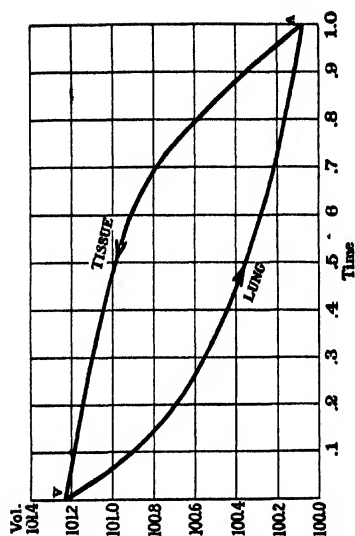


FIG. 115.

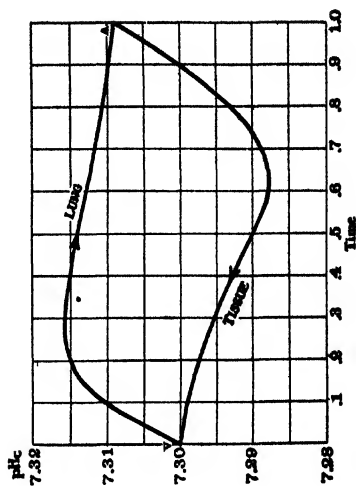


FIG. 117.

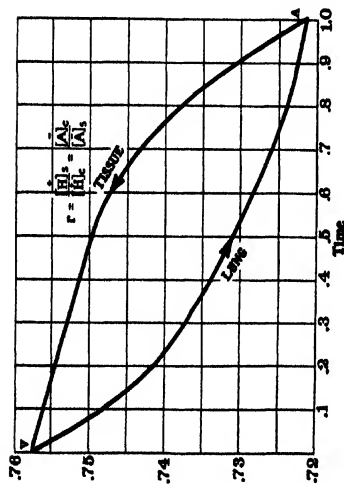
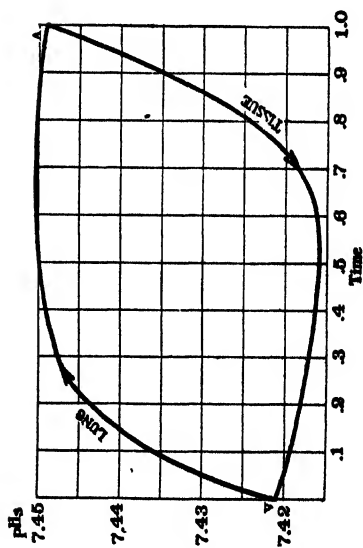


FIG. 116.



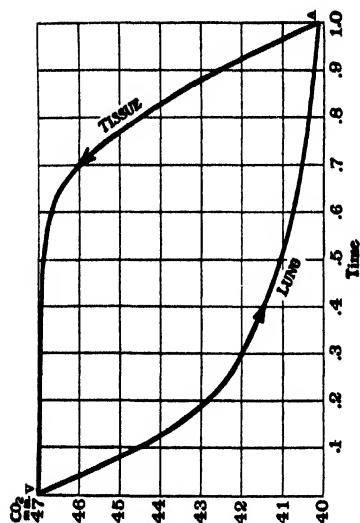


FIG. 119.

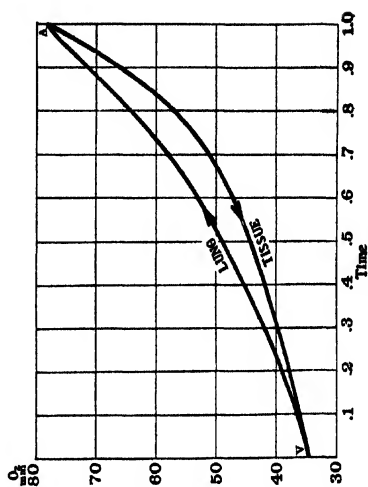


FIG. 121.

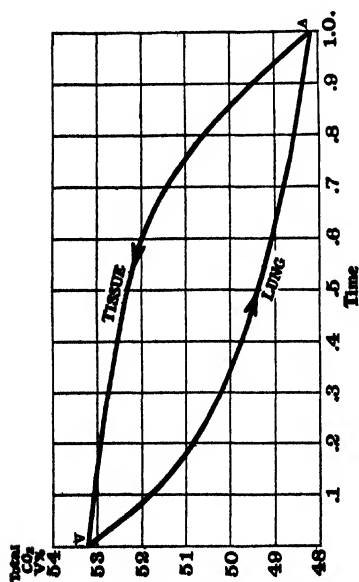


FIG. 118.

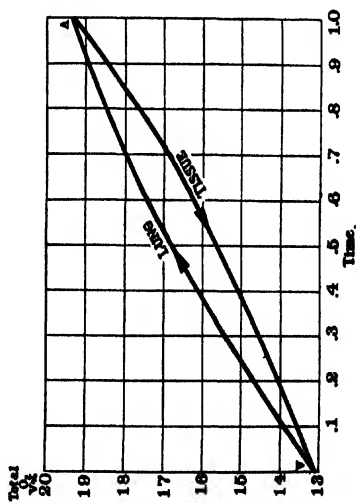


FIG. 120.

SUMMARY.

This paper aims to give a complete description of the changes known to occur in blood during the respiratory cycle and of the physicochemical system which determines them.

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THE EFFECT OF INSULIN UPON THE REDUCING SUBSTANCE IN THE CEREBROSPINAL FLUID OF NORMAL RABBITS.

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(Received for publication, January 17, 1924.)

It was first reported by Dèschamps and Bussy (1852) that reducing substance is usually found in the cerebrospinal fluid. Since that time numerous articles have appeared on this subject and recent investigations have proved definitely that the reducing substance is glucose, and that it is always present in the spinal fluid in normal conditions.

The sugar content of normal cerebrospinal fluid varies between 0.06 and 0.07 per cent. There is a considerable increase in the sugar content in diabetes mellitus and diabetic coma. Foster¹ found the sugar in the fluid of twelve cases of diabetes to vary between 0.5 and 3 per cent.

Since the Toronto workers proved in their experiments that insulin lowers the blood sugar in normal and diabetic animals, many studies on the determination of the sugar in the blood after subcutaneous administration of insulin have appeared, but no study on the determination of cerebrospinal fluid has been reported.

In the present work we have studied the effect of insulin on the liquor sugar of normal rabbits.

EXPERIMENTAL.

Rabbits were used in this investigation. The animals used were not fed during the experiment. The analytic method used

¹. Foster, cited by Levinson, A., Cerebrospinal fluid in health and in disease, St. Louis, 1919, 183.

to estimate the reducing substance in the cerebrospinal fluid was Bang's new micro method. In order to secure the cerebrospinal fluid from the subarachnoid space of the rabbit, a puncture needle is introduced into the space between the atlas and occipital bone. The skin must be previously cleansed. The needle is introduced in the median line in a slightly upward direction after passing

TABLE I.
Result of Examination of Cerebrospinal Fluid (Insulin).

Rabbit.		Body weight.	Interval after insulin injection.	Glucose in fluid.
No.	Sex.			
		<i>gm.</i>	<i>min.</i>	<i>per cent</i>
1	♂	1,580	30	0.051
2	♀	1,900	30	0.054
3	♀	2,280	30	0.051
			<i>hrs.</i>	
4	♀	2,190	1	0.048
5	♂	1,900	1	0.057
6	♂	1,920	1	0.052
7	♀	1,660	1	0.048
8	♂	2,520	1	0.056
9	♂	1,630	1	0.054
10	♀	2,170	2	0.030
11	♀	2,170	2	0.037
12	♂	1,700	3	0.002
13	♂	2,100	3	0.001
14	♂	1,760	3	0.017
15	♂	2,050	3	0.019
16	♀	2,250	4	0.035
17	♂	2,240	4	0.036
18	♂	1,500	5	0.040
19	♂	1,500	5	0.036
20	♂	1,640	6	0.055
21	♂	2,140	6	0.048
22	♂	2,180	7	0.059
23	♀	1,990	7	0.062

through a rather tough intervening tissue, the sudden cessation of resistance makes known the entrance of the point of the needle into the subcerebellar cisterna and then the spinal fluid is allowed to escape, or drawn off to the amount, as a rule, of 1 cc., slowly, but continuously. The fluid was taken by an occipito-atlantoid puncture at intervals ranging from 30 minutes to 7 hours after a

subcutaneous injection of insulin (Connaught Laboratories) in doses of 1 unit per 2 kilos of body weight, and carefully analyzed, as Table I and Chart 1 show.

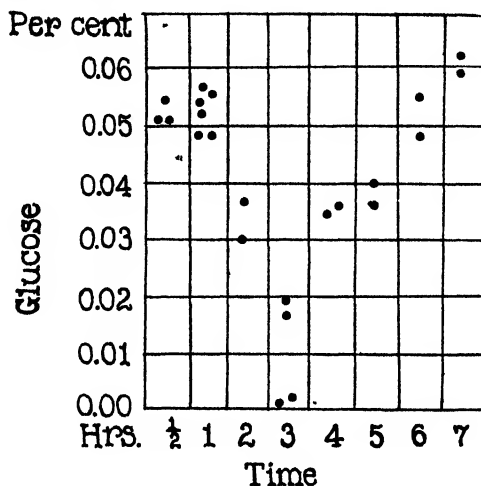


CHART 1.

TABLE II.

Glucose in the Cerebrospinal Fluid of Normal Rabbits.

Rabbit No.	Glucose.
	<i>per cent</i>
1	0.057
2	0.057
3	0.050
4	0.051
5	0.060
6	0.058
7	0.054
8	0.057
9	0.052
10	0.051

The control tests were done with cerebrospinal fluid taken from normal rabbits for this purpose, as Table II shows.

The amount of glucose in the cerebrospinal fluid of normal rabbits varied between 0.05 and 0.06 per cent. In the instance

of the 30 minute withdrawals after subcutaneous injection of insulin the fluids were in a normal condition. In the fluid examined 1 hour later only two out of six cases showed a slight decrease of sugar content. Hypoglycorachia (the decrease of the reducing substance in the cerebrospinal fluid) attained its maximum between the 2nd and 3rd hour. 7 hours later the fluid had returned to the normal condition.

From a consideration of the foregoing facts it can be assumed that there is probably in the cerebrospinal fluid and blood always some parallelism of the content of the reducing substance and that, therefore, its concentration in the fluid varies directly with the degree of glycemia.

SUMMARY.

There is a decrease in the concentration of the reducing substance in the cerebrospinal fluid after subcutaneous administration of insulin. There is some parallelism of the concentration of the reducing substance both in the fluid and blood after the administration of insulin.

THE DETERMINATION OF HYDROGEN IONS IN THE GASTRIC CONTENTS.

By J. F. McCLENDON.

(From the Laboratory of Physiological Chemistry, University of Minnesota Medical School, Minneapolis.)

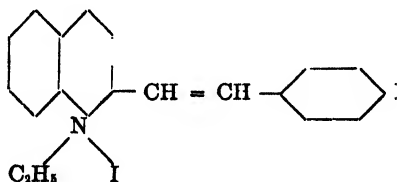
(Received for publication, January 30, 1924.)

Ever since the discovery of hydrochloric acid in the gastric juice by Prout, various methods have been used for the determination of this hydrochloric acid either in the pure juice or in the gastric contents. Of methods that have been recommended, the use of Gunzburg's reagent might seem the furthest from our present methods of titration since it involves the evaporation of the juice almost to dryness. In fact, there are probably many indicators preferable to Gunzburg's reagent. Töpfer's reagent is an indicator that may be used, and yet, it would probably not be selected by chemists at the present time. Methyl orange is a valuable indicator. To some persons, however, the color change of brom-phenol blue may seem a little more striking, and, therefore, we use brom-phenol blue in the titration of the gastric contents.

Ever since a comparison was made between acetic and lactic fermentations *in vitro* and gastric digestion, the idea has been expressed or implied that the gastric contents contain other acids than hydrochloric. From what we know now of dissociation constants of weak acids, and particularly of such polyvalent acids (ampholytes) as proteins, an attempt to titrate "the total acidity" of the gastric contents may seem somewhat futile. Even the process of titrating gastric contents back to a hydrogen ion concentration corresponding to the ingested food may seem of less meaning on close scrutiny than at first sight. If the food were placed in a beaker, its hydrogen ion concentration determined, the gastric juice added, and the mixture titrated to the original hydrogen ion concentration, a measure of the amount of acid in the gastric juice would be obtained, but in the stomach, saliva is

continuously poured into this mixture and at certain times duodenal contents are regurgitated. Whereas we may titrate the gastric contents to any desired pH, any titration of "total acidity" is impossible to interpret in quantitative chemical terms. It is therefore hoped that the term "total acidity" of the gastric contents will be dropped from our vocabulary.

Since it was shown by Sørensen and others that it is the pH of the gastric contents which is of significance in the digestion of food, the determination of the pH might seem advisable in routine gastric analysis if the technique is not made too difficult. It is the purpose of this paper to describe a technique which is fairly accurate, which is extremely rapid, and which requires only that apparatus which should be found in every hospital laboratory. The principle of the method is the determination of the percentage dissociation of an indicator by means of the colorimeter. The only valuable indicator which I have found so far for this purpose is quinaldine red.¹



It is intended to use the colorimeter already in the laboratory. It seems evident, however, that the principle used in the Duboscq has not been supplanted by anything better. There are several instruments on the market using this principle which do not use the Duboscq name. The colorimeter designed by Bürker² uses the same principle in matching the fields as used in the Duboscq, but by a different prismatic system made up of a single piece of glass. The apparatus is more rugged. The use of four cups enables the compensation for cloudiness in the gastric contents.

The following account is worded for the use of the small size colorimeters of the Duboscq type. Perhaps the Ewald test meal is very admirably designed for the titration of hydrochloric acid. It is not necessary to use any particular test meal with

¹ Eastman Kodak Co., No. 1361.

² Bürker, K., *Z. angew. Chem.*, 1923, xxxvi, 427.

this method. If it is desired to test the digestive power of the stomach, a meal containing sufficient nourishment should be used, perhaps the meal that is customary for the individual, or some standard meal of, for instance, 1,000 calories, containing about 35 gm. of protein. The drinking of coffee or of other dark liquids should be excluded. After drawing a sample, it is centrifuged, and the middle portion sucked up with a pipette. 5 cc. of this are placed in the left-hand cup of the colorimeter together with 3 drops of 1 per cent quinaldine red in 95 per cent alcohol, and mixed. The cup is set at 10 mm. (distance between plunger and bottom of cup). In the right-hand cup are placed 10 cc. of distilled water and 6 drops of quinaldine red, and after mixing, some may be transferred to a beaker if necessary. Under the right-hand cup is held a cup with black sides and clear bottom, 10 mm. in depth, filled with the gastric contents without the indicator. The color is now matched by moving the right-hand cup. The reading on the right-hand side in tenths of a millimeter gives the per cent of dissociation of the quinaldine red in the gastric contents. This figure is then found on the left-hand column of Chart 1. For instance, if this is 20, the horizontal line marked 20 is traced to the right until it intersects the diagonal line. It is then traced upward and the pH read on the top, which is in this case, 2.1. If the reading is 50, the pH is 2.7. This means that one-half of the indicator is dissociated. The dissociation constant is $10^{-2.7}$ according to these observations and the formula:

$$\frac{[H^+] \times [\text{Indicator}^-]}{[\text{H Indicator}]}$$

$= k$ becomes $\frac{10^{-2.7} \times \frac{1}{2}}{\frac{1}{2}} = 10^{-2.7}$. To show the limits of the method it is easy to distinguish the color change from a setting of 0.3 to 0.4 mm. of the right-hand cup which should mean a change in pH from 1.2 to 1.3. It also would be easy to distinguish the color change from a setting of 4.4 to 5.0 mm. which would denote a change in pH from 2.6 to 2.7. In the upper part of the chart the sensitivity per 0.1 on the pH scale is about uniform. In the lower part of the chart, however, the sensitivity decreases because of the fact that the percentage change of color per unit pH decreases. In the distinguishing of color the sensitivity depends upon the percentage change according to Webber's law within certain limits. For instance, we can easily dis-

tistinguish between 5.0 and 5.6 mm. in the setting of the right-hand cup which denotes a pH change of 2.7 to 2.8, but we cannot as easily distinguish a color change between 9.8 and 9.9 mm. in the setting of the right-hand cup, which denotes a change of 4.4 to

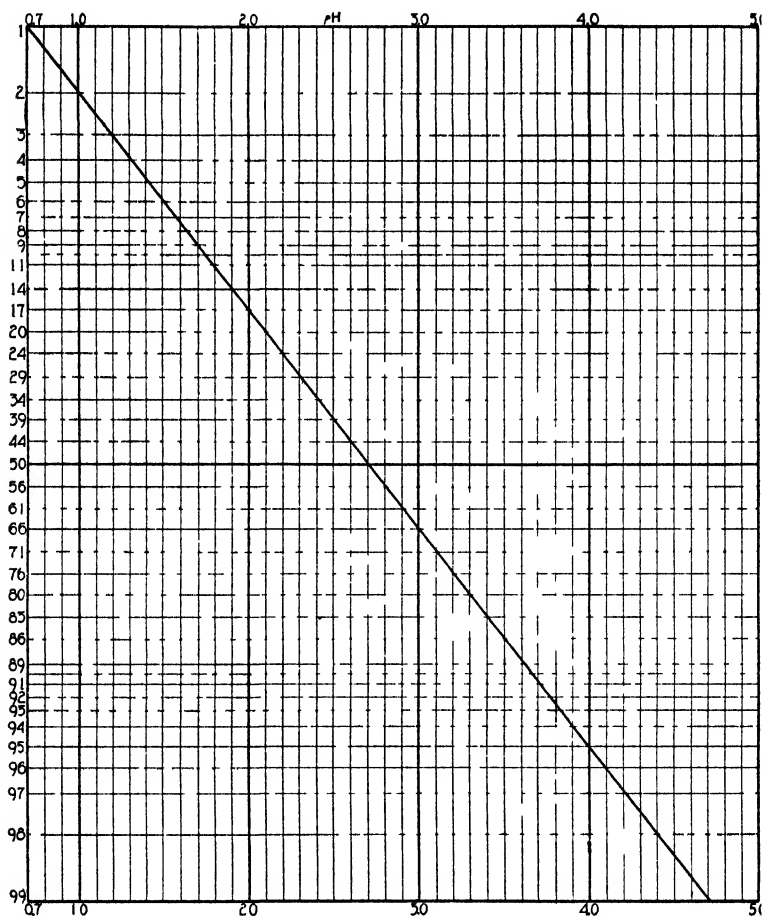


CHART 1

4.7 in the pH. In fact, if our eyes are sensitive only to a 5 per cent change in color, the best we can do is to distinguish between 9.4 and 9.9 in the setting of the right-hand cup which denotes a pH change of 3.9 to 4.7. Since, however, a titratable amount of

hydrochloric acid in the gastric contents would denote a pH between 1 and 4, about all we can say as a result of this examination, if the reading of the right-hand cup is greater than 9.5, is that there is no free hydrochloric acid in the gastric contents. Since many foods and drinks contain acetic and lactic acids, and often small quantities of phosphoric acid, the determination of the pH of the adult's stomach between 4 and 7 is difficult to interpret. It has already been shown³ that most foods are acid. In fact, with the exception of a few alkaline mineral waters, and milk immediately from the lacteal glands, perhaps all foods and drinks are acid. Therefore, the method is not designed for the detection of these very slight acidities. The mode of construction of the chart has already been described.⁴ The advantage of this method is that by making the curve a straight line, new curves are easily drawn. They should be drawn parallel to the diagonal line on the chart, which can easily be done by means of parallel rules. The addition of salt will change the dissociation constant of quinaldine red, and it is merely necessary to find the logarithm of the reciprocal of the dissociation constant under the given conditions, and having determined this on the pH scale, trace this coordinate to the 50 per cent dissociation line and draw the diagonal through the point of intersection. If the dissociation constant is not determined, all that is necessary is to determine a single value of pH corresponding to a known percentage dissociation, find this point on the chart, and draw the diagonal through this point. It is possible that the admixture of some other red dye in the quinaldine red as an impurity might be dealt with approximately by determining a single point on the chart, and drawing a new diagonal in this way.

By means of the micro colorimeter made by Bausch and Lomb, it is possible to make this determination on 0.5 cc. of gastric juice in the left-hand cup together with 0.5 cc. of gastric juice or fluid of equal opalescence in a cup held beneath the right-hand cup.

Phosphates shift the average wave-length of the color of quinaldine red, and hence if the gastric contents contain a high concentration of phosphates, it is necessary to put some alkaline phos-

³ McClendon, J. F., and Sharp, P. F., *J. Biol. Chem.*, 1919, xxxviii, 531.

⁴ McClendon, J. F., *J. Biol. Chem.*, 1922, liv, 647.

phates in the distilled water standard. I have not encountered a sufficiently high concentration of phosphate in any sample of gastric content so far studied to cause detectible effect on the color.

**CAROTIN—THE PRINCIPAL CAUSE OF THE RED AND
YELLOW COLORS IN PERILLUS BIOCULATUS (FAB.),
AND ITS BIOLOGICAL ORIGIN FROM THE LYMPH
OF LEPTINOTARSA DECEMLINEATA (SAY).***

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It has been shown by one of us¹ that the variation in the hypodermal color pattern of the stink-bug, *Perillus bioculatus* (Fab.), from white and black to red and black (with various intermediate yellow and black forms) is not due to inheritance but to variations in the physiological activity of the insects. Temperature was used to control the physiological activity in the experiments referred to, high temperature causing changes in color pattern from red and black to white and black in the immature stages and low temperature causing the deposition of yellow to red pigment in all life stages including the adult. Physiological activity as expressed in sexual functions, such as egg-laying, was also found to influence the color pattern in the adult females, retarding the deposition of red pigment in the body walls.

Another point of great interest in connection with *Perillus* is the fact that the food of the adult insects as well as of the various nymph stages, except the first, consists almost exclusively of the eggs and larvæ of the potato-beetle (*Leptinotarsa decemlineata* Say) as well as the adult beetle itself. This food is always highly colored with orange-yellow pigment. Moreover, when the larvæ and adult beetles are attacked the golden yellow lymph only is eaten.

* Published with the approval of the Director as paper No. 429 of the Journal Series of the Minnesota Agricultural Experiment Station.

¹ Knight, H. H., 19th Report, State Entomologist of Minnesota, 1922, 50.

The two facts brought out in the above mentioned study on *Perillus*, namely a constancy of yellow pigment in the food and variations in the utilization of the pigment with alterations in physiological activity, suggested a study of the chemical nature of the food pigment and of the pigment deposited in the hypodermis. It was seen that the identification of the pigment would at once establish its character both for the stink-bug and for its host, the potato-beetle. Such a study also gave promise of establishing for the first time the biological origin of an insect pigment in a manner analogous to the identification of the source of carotinoids in cattle and fowls in an earlier experiment by one of us.²

EXPERIMENTAL.

The results of the chemical study of the red and yellow pigment in the stink-bug and its host were as follows:

Carotin in the Potato-Beetle Larvæ.—The chemical study was limited to the lymph inasmuch as this fluid is the only portion of the larvæ used as food by its predaceous enemy. The lymph was removed from about 200 full grown larvæ by pricking with a needle and allowing the golden-yellow fluid to flow out without being contaminated by substances of a fatty nature or by ingredients from the digestive tract. The fluid was dried at once on anhydrous Na_2SO_4 , giving a rich orange colored powder, which was used for the solubility tests.

It was found that petroleum ether did not remove the pigment from the pigmented Na_2SO_4 to any appreciable extent until the salt was first moistened with alcohol, after which the pigment could be removed quantitatively. Ether or carbon disulfide readily extracted the pigment although the extraction was not complete in the case of carbon disulfide until the pigmented Na_2SO_4 was first treated with alcohol. The ethereal solutions of the coloring matter were golden-yellow and the carbon disulfide solution rose-red. No evidence whatever of xanthophyll-like pigments could be obtained by shaking the petroleum ether or carbon disulfide solutions with 80 per cent methyl alcohol,

² Palmer, L. S., and Eckles, C. H., *J. Biol. Chem.*, 1914, xvii, 191, 211, 223.
Palmer, L. S., *J. Biol. Chem.*, 1915, xxiii, 261.

the pigment in each case remaining quantitatively in the original solvent. These solutions also showed the spectroscopic properties of carotin.*

The deep orange color of the potato grub lymph suggested a quantitative determination of the concentration of carotin in the fluid. This was made on a fresh portion of lymph carefully prepared from about 190 grubs. 5 cc. of the lymph were transferred to a small separatory funnel with a pipette, and, after dilution with 10 cc. of water, shaken with 50 cc. of ether. This extracted nearly all the pigment, but in order to render the lymph colorless the extraction was repeated with two additional 25 cc. portions of ether after first adding 5 cc. of methyl alcohol to the diluted lymph. The ether solution of pigment was diluted to exactly 100 cc. and its color compared with a standard 0.20 per cent solution of $K_2Cr_2O_7$ in a Duboseq colorimeter. The amount of carotin present was then calculated according to the procedure suggested by Palmer.⁴ Since 100 mm. of the dichromate were found to be equal in color to 25.1 mm. of the lymph extract it was estimated that the extract contained approximately 0.00068 per cent carotin, which would be equivalent to 0.0136 per cent carotin in the lymph. This is an astonishingly high concentration of carotin for an animal fluid and equals that found in many fresh green leaves.

Carotin in Perillus bioculatus (Fab.).—It being desired to determine the character of the yellow and red pigment in the body wall, the bugs were prepared for extraction by carefully dissecting away the intestines, eggs and all free lymph in the abdominal and thoracic regions leaving only the hypodermis and its chitinous exoskeleton. 50 fresh bugs were thus prepared.

* It is a matter of great interest that not a trace of xanthophyll could be found in the highly colored lymph of the potato-beetle. There can be hardly any doubt that the green potato plant is as rich in xanthophylls as other green plants, and the leaves are also said to contain lycopin. It is surprising to find that this insect absorbs carotin and excludes all the other carotinoids in its food. The discovery of the mechanism by which the insect does this might give a clue as to how the higher animals, e.g. the cow and the horse, eliminate the xanthophylls and absorb only the carotin from their food.

⁴ Palmer, L. S., Carotinoids and related pigment. The chromolipoids, New York, 1922, 260.

It was found possible to extract a large amount of pigment from the bugs by macerating them in a mortar with carbon disulfide or petroleum ether, giving deep orange-red and golden-yellow solutions, respectively. No xanthophyll could be detected by shaking these extracts with 80 per cent methyl alcohol. Both solutions showed two distinct absorption bands, although in the case of the carbon disulfide solution the bands were not as brilliant as one usually sees with a fresh carotin extract from plant tissue. The concentrated carbon disulfide solution was blood-red in color, and freshly evaporated, solvent-free residues on white porcelain surfaces were very deeply pigmented. It is thus evident that the yellow and red colors seen in the body wall of the stink-bug *Perillus* and the beetle *Leptinotarsa* are due to differences in concentration of the same pigment. Such a color variation is, in fact, a characteristic property of carotin. The pigment residues also readily gave the green color reaction with ferric chloride which is characteristic of carotinoids.

The hypodermis of the bug is not, however, devoid of water-soluble coloring matter. Hot water readily extracts yellow pigment from the macerated fresh tissue, giving extracts which deepen to orange on addition of NH_4OH and whose original color is restored by acid. The latter property suggests a flavone-like substance. It is probable, also, that this pigment plays a minor part in the hypodermal coloration because the black and white forms of the bug do not yield any appreciable amount of color with hot water. These extracts, however, show some color on addition of alkali, indicating the presence of traces of the flavone-like pigments.

DISCUSSION.

It seems to us that the identification of the variable yellow and red pigment of *Leptinotarsa* and *Perillus* as carotin and the establishment of its origin as a food derivative in the case of these two orders of insects are of considerable biological importance. We wish to discuss these findings from two points of view: (1) as affording an explanation of the variations in yellow and red pigmentation with changes in the environment; and (2) as furnishing a vulnerable point of attack upon the probability that these color variations can become fixed and thus inheritable.

Assuming that all experimental changes in environment with *Leptinotarsa* and *Perillus* are carried out with a constant food supply, i.e. potato plants for the beetle and potato-beetles for the stink-bug, it is at once significant that the insects are continually supplied with the carotin which is deposited in varying quantities in the hypodermis. It is therefore obvious that only black and white insects of both orders would be produced, regardless of the environment, if the insects could be reared on carotin-free food. In the experiments on *Leptinotarsa* by Tower,⁵ and on *Perillus* by Knight,¹ the principal environmental changes which brought about the variations in pigmentation were changes in temperature. Since the activity of insects is closely correlated with the temperature of their environment and since *Leptinotarsa* and *Perillus* both show the same kind of variations in color with temperature, it seems reasonable to believe that the conclusion of one of us,¹ that the pigment variations in *Perillus* are due to changes in physiological activity induced by temperature changes, should be extended to include the pigment variations in the potato-beetle whose yellow pigment is the same chemical substance. As a matter of fact it will be seen from the following facts that the identification of this pigment as carotin furnishes convincing evidence of the correctness of this view. (1) All experimental evidence so far secured points to the fact that carotin is to be regarded as a fortuitous substance in animal forms, to be got rid of as best the animal may. Being a readily oxidizable substance it seems reasonable to assume that oxidation is one of the normal paths of elimination. Deposition of carotinoids in the epidermis is frequently seen in the higher animals and may also be considered a method of elimination. (2) It is of interest, and perhaps closely analogous to the deposits of carotin in *Perillus* and no doubt *Leptinotarsa* at low temperature, that the appearance of carotin in the epidermis in man occurs most frequently in diabetics on a carotin-rich diet when the oxidative tone of the body is subnormal. It seems reasonable to suppose, therefore, that the physiological activity of the insects which is modified by the temperature is oxidation, influenced by the physical activity of the insects, and that this is the fundamental explanation of the pigment variations.

⁵ Tower, W. L., Carnegie Inst. Washington, Pub. 48, 1906, 259.

One fact which is surprising in connection with the red pigment deposits in *Perillus* is the great stability of the carotin when once deposited in the adult bug. The carotin is no longer removed by raising the temperature and the mounted specimens retain their red color, apparently indefinitely. These facts, when coupled with the knowledge of the character of the pigment, suggested that the carotin might be bound to protein in the hypodermis and thus protected. No evidence of such a union could be found, however, and the conclusion has been drawn that the protection of the pigment from oxidation in the adult hypodermis is wholly structural.

It is well known to those interested in genetics that Tower⁵ has presented evidence indicating that white and black forms of *Leptinotarsa decemlineata* (Say) produced by environmental changes would breed true, thus presenting a case of inheritance of an acquired characteristic. As already stated, one of us¹ has been unsuccessful in repeating these observations using *Perillus bioculatus* (Fab.), the white and black modifications breeding true only when the environment which first produced them was maintained. It seems very easy to understand why this result was secured when it is considered in the light of the character and origin of the modifiable pigment of *Perillus*. It is important to note, however, that the origin of the red *Perillus* pigment is the lymph of the potato-beetle, *Leptinotarsa decemlineata* (Say), which must, *ipso facto*, supply the pigment for its own hypodermis. With these facts in mind it becomes at once a matter of great importance for both biochemistry and genetics to substantiate Tower's results.

From the biochemical point of view it would appear that Coleoptera can readily modify permanently the simple physiological functions which dispose of the carotin taken in with the food, whereas Hemiptera cannot do so, a proposition which is difficult to accept without further substantiation. In fact, the identification of the yellow pigment of *Leptinotarsa* as a food pigment without the evidence on *Perillus* would raise a question in the minds of the biochemist as to the correctness of Tower's results.

From the genetic point of view it is well known that Tower's data have been accepted by many as establishing a clear-cut

case of inheritance of acquired characters. When viewed from the angle furnished by the chemical identification of the pigment and the negative results secured in a study of an analogous case in another order (which feeds on *Leptinotarsa*) this example of inheritance deserves renewed attention. It is admitted that mutations might occur in which carotinoids fail to be absorbed,⁶ but it must certainly be regarded as an open question that such mutations *can be produced by environment*.

CONCLUSIONS.

1. The yellow and red colors seen in the hypodermis of the stink-bug, *Perillus bioculatus* (Fab.), are due largely to carotin which is derived from the food; namely, chiefly the lymph of the potato-beetle, *Leptinotarsa decemlineata* (Say).

2. The lymph of the potato-beetle, *Leptinotarsa decemlineata* (Say), is colored exclusively by carotin, the concentration amounting to 0.0136 per cent in the fresh lymph, which is as high as is encountered in fresh green leaves.

3. Experimental data purporting to show that the amount of carotin deposited in the hypodermis of insects can be permanently modified by environment without removing the source of pigment require substantiation before they may be accepted as illustrating the inheritance of acquired characters.

⁶ See Gerould, J. H., *J. Exp. Zool.*, 1921, xxxiv, 385, for an account of such a mutation in a normally carotinoid-absorbing caterpillar.

**ANTHOCYANIN AND FLAVONE-LIKE PIGMENTS AS CAUSE
OF RED COLORATIONS IN THE HEMIPTEROUS
FAMILIES APHIDIDÆ, COREIDÆ, LYGÆIDÆ,
MIRIDÆ, AND REDUVIIDÆ.***

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(Received for publication, December 26, 1923.)

It has been shown by us¹ in a previous paper that the red and yellow colors seen in the stink-bug, *Perillus bioculatus* (Fab.), are caused largely by carotin, derived from its food which consists almost wholly of the highly pigmented eggs and larvæ of the potato-beetle (*Leptinotarsa decemlineata* Say) as well as the mature beetle, which, in turn, derives the carotin from the carotin-rich potato plant. This finding suggested the chemical examination of the red pigment exhibited in several other species of the Hemiptera, particularly phytophagous forms. The results were as follows:

Tritogenaphis rudbeckiæ (Fitch), (*Macrosiphum* Authors), Family Aphididæ—This bright vermilion colored aphid, which inhabits the stems of golden-glow, yielded small amounts of color when several hundred of the fresh bugs were macerated in a mortar and treated with petroleum ether or carbon disulfide. The latter solution was rose colored and exhibited two well defined absorption bands in the green and blue region of the spectrum, and left a residue on evaporation which gave the usual green carotinoid color test with ferric chloride. Inasmuch as the petroleum ether extract failed to show the presence of xanthophylls when shaken with 80 per cent methyl alcohol, the con-

* Published with the approval of the Director as paper No. 430 of the Journal Series of the Minnesota Agricultural Experiment Station.

¹ Palmer, L. S., and Knight, H. H., *J. Biol. Chem.*, 1924, lix, 443.

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clusion seems justified that the pigment which can be extracted by fat solvents from the aphids consists exclusively of carotin.

The major red pigment in the aphids was not soluble in fat solvents but was readily extracted by water or cold methyl alcohol, yielding vermilion colored solutions. The red color of these solutions changed to green on addition of ammonium hydroxide and the original red color was restored by acid. When solutions of the vermilion pigment were evaporated to dryness (after first freeing the alcohol extract of carotin by shaking with petroleum ether) decomposition occurred; the color changed to a brownish red and the residue was no longer completely soluble in water. This residue, however, gave a deep green color test with ferric chloride. The properties of the vermilion pigment strongly suggest an anthocyanin.

Leptocoris trivittatus (Say), Family Coreidae.—This red and black patterned box-elder plant-bug inhabits chiefly the stems of its host, deriving its food from the sap. The lymph and abdominal cavity were dissected out of 75 fresh bugs and the hypodermis, containing the red pigment, macerated with sea sand. Attempts to extract the red pigment with petroleum ether, ether, carbon disulfide, methyl alcohol, or hot ethyl alcohol apparently resulted in failure, although when these extracts were combined and evaporated there was evidence of traces of pigment of a carotinoid nature. The principal red pigment in the hypodermis readily dissolved in warm water, however, giving a golden-yellow solution. This color was deepened considerably on addition of alkali and the original golden color was restored by acid. The property of color change as well as the water solubility of the pigment suggests a coloring matter of the flavone type.

Lygæus kalmii (Stål), Family Lygæidae.—Twenty-five dried specimens of these red and black patterned milkweed plant-bugs were crushed with sand in a mortar. It was not possible to extract any pigment from this material with the fat solvents or with alcohol, but hot water readily gave a bright yellow extract whose color deepened on addition of alkali.

Lopidea staphylææ sanguinea (Kngt.), Family Miridae.—Forty-five dried specimens of these red and black (chiefly red) patterned bladdernut plant-bugs were crushed and treated with various solvents. Hot water, only, removed the pigment, giving golden colored solutions.

Coccobaphes sanguinarius (Uhler), Family *Miridae*.—Fifteen dried specimens of this chiefly red form which feeds on maple leaves and stems were crushed and treated with various solvents. Hot water, only, extracted the pigment giving golden colored solutions whose color deepened on addition of ammonium hydroxide and was restored to the original tint by acid.

Eulyes illustris (Stal), Family *Reduviidae*.—This large, chiefly predaceous bug of the Philippines, called the assassin-bug, was expected to be of special interest because we¹ had found the red pigment in the predaceous *Perillus* to consist exclusively of carotin. The single specimen examined, which was brilliantly colored, showed the presence of water-soluble pigment only. The color of the extract showed the deepening of color with ammonium hydroxide and the restoration with acid which was found for the extracts from most of the other forms examined in this series, and which resembles a flavone pigment.

DISCUSSION.

The results of the examination of the red pigment in this series of Hemiptera are of interest for several reasons. Primarily the tests indicate that water-soluble pigments rather than pigments of the carotinoid type predominate as the cause of the red color in phytophagous forms, and, therefore, in Hemiptera in general, inasmuch as predaceous families are in the minority among these insects. It is true that Phisalix² has apparently identified carotin as the cause of the red color in the chiefly phytophagous bug *Phyrrhocoris apterus* L., while we¹ have identified the same pigment in *Perillus bioculatus* (Fab.), a wholly predaceous form. It cannot be inferred from the latter, however, that the red colored predaceous forms, which in reality are not particularly abundant, always owe their color to carotin. The highly pigmented assassin-bug (*Eulyes illustris* Stal), which is predaceous, failed to show the presence of carotinoids.

Of the phytophagous forms examined it is interesting to note that carotin occurred in small amounts in the red golden-glow aphid and in the box-elder plant-bug. Our results show, however, that carotin contributes little if any to the visible red color

² Phisalix, C., *Compt. rend. acad.*, 1894, cxviii, 1282.

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of these bugs. That which was found may have been present in the digestive tract of the bugs which could not be removed because of their diminutive size.

A further point of interest is the presence of two types of water-soluble red pigments in the families examined. In the aphid the tests on the extracted material strongly resemble those of an anthocyanin. In the other forms the properties of the pigment suggest a flavone-like coloring matter. This result raises the question whether anthocyanin and flavone-like pigments in these insects are to be regarded as direct derivatives from the food, as carotin is now known to be, or whether they are to be considered as synthesized by these animal forms. In reply to this question it may be said that if these pigments should actually prove to be anthocyanins and flavones, the presumption would certainly be that they are derived from the food, either directly or indirectly as chromogens. In this case just as much doubt would be thrown upon the use of these types of color forms for genetic research as is now the case for the forms whose yellow or red color is due to derived carotin; *i.e.*, *Leptinotarsa decemlineata* (Say) and *Perillus bioculatus* (Fab.). When pigment in an animal species has once been shown to be independent of the synthetic powers of the protoplasm and subject to simple fundamental physiological processes, as seems to be the case for the red pigment of the potato-beetle and the stink-bug *Perillus*, grave doubt arises as to the significance of color variations affecting this pigment as indicating a possible inheritance of acquired characters unless it be admitted that the simple physiological processes of the animal can be permanently modified by environment.

CONCLUSIONS.

1. Red pigment in both phytophagous and predaceous families of Hemiptera is not limited to one type of substance. Water-soluble pigments appear to be more common than carotin.
2. The aphid *Tritogenaphis rudbeckiæ* (Fitch) owes its vermilion color chiefly to an anthocyanin-like pigment, although small quantities of carotin are also present in the bug.
3. The red color of the red and black patterned phytophagous box-elder plant-bug (*Leptocoris trivittatus* Say), the milkweed

plant-bug (*Lygaeus kalmii* Stal), the bladdernut plant-bug (*Lopidea staphyleæ sanguinea* Knegt.), the maple plant-bug (*Coccobaphes sanguinarius* Uhler), and the predaceous assassin-bug (*Eulyes illustris* Stal) is due to a flavone-like pigment.

4. It is difficult to understand how environment can cause a permanent modification of an insect color pattern involving an animal pigment which is derived from the food, and which is subject to fundamental physiological processes of the protoplasm, without first causing a permanent modification of the processes to which the derived pigment is subject. The assertions that red pigment in insects can be thus modified and the modification become inheritable seem to lose weight in the light of the finding that the pigment is likely to be a carotinoid, anthocyanin or flavone, derived from the food.

POLARIMETRIC OBSERVATIONS ON SOLUTIONS OF GLUCOSE AFTER CONTACT WITH THE INTESTINAL MUCOSA.

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In 1920 Hewitt and Pryde (1) published the results of a series of experiments in which they described a remarkable mutarotation noted when solutions of glucose were allowed to remain for a few minutes in contact with the intestinal mucosa of the rabbit. This change consisted in a rapid downward mutarotation of the sugar solution to optical values corresponding to specific rotations much below $+52.5^\circ$, while after withdrawal from the intestine these solutions were observed to undergo a slower upward mutarotation to a permanent value corresponding with the specific rotation of α - and β -glucose in equilibrium. These results were interpreted by Hewitt and Pryde as indicative of the formation from glucose of the unstable and highly reactive stereoisomer γ -glucose. The findings of Hewitt and Pryde are of far reaching importance in the study of carbohydrate metabolism as indicated, for instance, in the adoption of their theories by Winter and Smith (2) in their recent work on the nature of the sugar in normal and in diabetic blood; so important in fact (if we consider the great variety of biochemical problems in connection with which such an hypothesis might be utilized), that it seemed desirable to repeat and, if possible, extend this work. The results of such a repetition have recently been published by Stiven and Reid (3) who have been entirely unable to confirm the findings of Hewitt and Pryde as they report that in twenty-six experiments they were in no instance able to detect any sign of mutarotation in the earlier minutes after removal of the solution from the intestine.

In view of the fact that the results of Stiven and Reid are entirely at variance with those of the earlier investigators, it seemed to us that a repetition of the work of both would be very much worth while. We have confined our investigations to the action of the mucosa of the small intestine on *d*-glucose; and, in carrying out this work have adopted every possible measure that would assure uniformity of procedure.

Procedure.

Rabbits which had been fasted for 24 hours were employed as experimental animals. Anesthesia was induced with ether, administered in the beginning of the experiment by cone, and later by means of a tracheal cannula. Throughout the course of each experiment the animal was kept warm by an electrically heated aluminum plate which formed the bed of the operating table. Glass cannulas were inserted in the small intestine just below the pyloric end of the stomach, and above the ileocecal valve, the average length of intestine used being 74 inches. The nerve and blood supply of the intestine were kept intact, and Tyrode's solution previously warmed to 38° was used in washing out debris, small shreds of intestinal mucosa, and such other material as might subsequently render the glucose solution difficult to filter. In two instances large tapeworms were found. To insure that as much as possible of the intestine should come in contact with the glucose solution, the volume of Tyrode's solution necessary to fill the intestine, avoiding distention, was measured roughly, and, after all but traces of the Tyrode solution had been drained off, twice this volume of glucose solution, warmed to 38°, was passed through the intestine, the last portion being allowed to remain in the intestine for 10 minutes. This procedure reduced to a minimum the dilution of the glucose solution by irrigation fluid which had been retained in the intestine.

The intestine was handled as little as possible during the contact period, and was kept within the abdomen, the incision in which was covered with gauze soaked with Tyrode's solution kept warm by means of an electric bulb suspended a few inches above the animal.

●

For filtering the glucose solutions, we used a pair of Gooch crucibles with asbestos mats built up on a disc of filter paper, and to prevent disintegration of the asbestos mat, loose pads of absorbent cotton moistened with 2 per cent glucose solution were placed on the mats and full suction from a small water pump was applied before any liquid was placed in the crucibles. In twenty-four experiments with this system, the average time which elapsed from the removal of the glucose solution from the animal to the first polariscopic reading was 5.9 minutes. In two instances the first reading was obtained in 3 minutes, but in four experiments where it was necessary to use alumina cream to get a filtrate clear enough for observation, the time required was 9 to 11 minutes. The filtrates obtained in three experiments were discarded as being too opalescent to permit accurate adjustment of the instrument. The time involved in the various steps in our procedure was kept account of by means of two watches, one in the dark room the other in the operating room, both being set together.

Polarimetry.

In our investigations we used a Schmidt and Haensch three-field instrument of the Lippich type, reading direct to 0.01° and Schmidt and Haensch 2 and 4 dm. tubes. Readings were taken at $22-25^\circ$ and to preserve the dark adaptation of the observer's eye as far as possible, scale readings on the instrument were made with a small shielded flash-light. Preliminary experiments indicated that sodium light was not sufficiently intense to allow adjustment of the field of the instrument within the observer's known error on stable aqueous glucose solutions, and since the determination of whether or not any mutarotation occurs in the glucose solution immediately after contact with the intestinal mucosa, was of more importance in the problem under investigation than the accurate determination of the ratio between the polarimetric and copper-reducing values of the glucose solutions, we have abandoned the use of sodium light entirely and in all our experiments have used a 100 c.p. electric bulb with a compact spiral filament as a source of light, and as a filter a 3 cm. layer of 3 per cent $K_2Cr_2O_7$.

In order to obtain greater sensitivity in polariscopic readings the observer entered the dark room at least 15 minutes before the time at which the first reading was to be made, the final injection of glucose into the intestine, filtration, etc., being carried out by the second worker who in no case attempted to make polariscope readings.

Solutions and Analytical Method.

The glucose solutions used in our work were made up from a product prepared by the Digestive Ferments Company of Detroit and marked "Difco Standardized, Anhydrous Glucose." This material dissolved quickly and completely to give clear solutions which were filtered and kept in an ice box for 10 days before being used which allowed ample time for the equilibrium between α - and β -glucose to be established. The concentrations of these solutions, as determined by the method and reagents of Shaffer and Hartmann (4), were 2.00 and 2.01 per cent, respectively, and they were found to have a pH of 5.2.

The pH of the polariscope tube contents was determined colorimetrically, using freshly prepared buffers which had been standardized electrometrically, and the copper-reducing value according to the technique of Shaffer and Hartmann (4).

The alumina cream, used in four experiments only, was prepared by adding a slight excess of ammonia to a saturated solution of $KAl(SO_4)_2$; the resultant precipitate was washed by decantation with distilled water for 8 days, and then washed further by repeated centrifuging. The supernatant liquid removed from the final product had a pH of 6.0.

In Table I we have collected the results obtained in twenty-one experiments in which we have attempted to repeat the work of Hewitt and Pryde and of Stiven and Reid, and as far as polarimetric evidence is concerned, it is evident that our observations are more in accord with the findings of the latter investigators than with those of the former.

Among the twenty-four experiments made, three results were obtained which might be termed inconclusive as the solutions removed from the intestine were discarded as being too opalescent to allow of accurate reading on the polarimeter, in twelve ex-

periments no sign of optical change was evident in the first 20 minutes after removal of the solution from the intestine, that is, no change within the observer's error of adjustment of the field of the polariscope, an error which was found in frequently repeated trials to be $\pm 0.02^\circ$. In these twelve experiments it was found that our results were also in accord with those of Stiven and Reid as regards the relation between the glucose concentration of the intestinal contents as determined by analysis and as calculated from the polariscopic readings, the glucose value being invariably higher when determined by titration than when calculated from the observed rotation. To eliminate the error that would be found in our calculated glucose concentrations, due to our not having used monochromatic light, we have determined the ratio between the concentrations of our original 2.00 and 2.01 per cent glucose solutions as determined by analysis, and by calculation, and have used this ratio as a basis of comparison. The explanation of this finding offered by Stiven and Reid, namely that levo-rotatory material is present in noticeable amounts in the intestinal secretion which becomes mixed with the glucose solution during its sojourn in the intestine, would appear plausible, although scarcely amenable to direct experimental proof, but the fact should not be overlooked that in all probability substances possessing the power to reduce copper solutions may also be absorbed from the intestinal mucosa.

In five of the remaining nine experiments a small upward mutarotation was observed while in four instances a far greater downward change was noted.

In four experiments it was necessary to use alumina cream to clarify the solution, and, so far as we can see, the addition of the reagent produced, aside from its clarifying effect, no change in the solution except to shift the pH towards the acid side, due no doubt to the fact that the alumina cream itself had a pH of 6.0. Three of our animals died just before we were ready to inject the glucose into the intestine, and in each case the experiment was carried through to completion in the usual way. The only change noted in these cases was the change of the reaction of the intestinal contents from the usual faintly alkaline or neutral reaction to a hydrogen ion concentration on the acid side.

Glucose concentration introduced into intestine, treatment of filtrate, etc.	Readings at 2 minute intervals,						
	3	5	7	9	11	13	15
2.00 per cent glucose, 2 dm. tube, alumina.....					1.48		1.35
2.00 " " " 4 " " no alumina..	4.13	4.08	4.00		3.91	3.89	
2.01 " " " 4 " " " " ..		3.96			3.92	3.90	
2.00 " " " 4 " " " " ..		3.00	3.05	3.07		3.09	
2.00 " " " 4 " " " " ..		3.90	3.93	3.92		3.96	3.95
2.00 " " " 4 " " " " ..			3.75	3.74	3.77		3.83
2.00 " " " 4 " " " " ..		4.05	4.04		4.09	4.09	4.10
2.01 " " " 4 " " " " ..		3.68	3.66	3.65	3.65		3.64
2.01 " " " 4 " " " " ..		2.32	2.34	2.36	2.37	2.38	
2.00 " " " 4 " " " " ..		3.19	3.20		3.21		3.21
2.00 " " " 4 " " " " ..		3.68	3.69	3.70	3.69		3.70
2.00 " " " 2 " " " " ..		1.50		1.50		1.52	
2.00 " " " 4 " " " " ..		4.15	4.14	4.15		4.16	
2.00 " " " 2 " " alumina....						0.85	
2.00 " " " 4 " " no alumina..	4.09	4.10		4.11	4.11		4.10
2.01 " " " 2 " " alumina.....					1.38	1.38	
2.01 " " " 4 " " "		2.48		2.47	2.49	2.48	
2.01 " " " 4 " " no alumina..		3.68		3.68		3.68	
2.01 " " " 4 " " " " ..		1.20		1.22		1.21	
2.01 " " " 4 " " " " ..		4.03		4.03		4.02	
2.01 " " " 2 " " " " ..				1.73			1.76
2.00 " " "							
2.01 " " "							

* Animal died before completion of experiment.

SUMMARY.

In a repetition of the work of Hewitt and Pryde and of Stiven and Reid on the production of mutarotation in glucose solutions by contact for brief periods with the mucosa of the small intestine of the rabbit the results in twenty-four experiments were divided as follows: in twelve cases no change in rotation was found, in five cases a small upward rotation and in four cases a somewhat greater downward change, while in three cases the

21	23	25	27	29	31	33	35	45	Maximum change in rotation in degrees.	Glucose concentration from copper-reducing value.	Glucose concentration from polariscope readings.	Cu value Ratio Polarimetric reading.	pH of filtrate.	Animal No.
1.10			1.00		1.02	"		1.01	-0.47	1.32	0.96	1.38		9
	3.88		3.90						-0.23	1.97	1.86	1.06		2
								3.83	-0.13	2.08	1.82	1.14	6.8	23*
3.11	3.11							3.11	+0.11	1.63	1.56	1.05		6
3.97	3.99						4.00		+0.10	2.02	1.91	1.06		8
	3.85		3.86					3.84	+0.09	1.86	1.84	1.01		4
								4.13	+0.08	1.79	1.97	0.91		11
								3.61	-0.07	2.10	1.72	1.22	7.3	16
								2.38	+0.06	1.15	1.13	1.02	7.4	17
		3.20							0.00	1.85	1.54	1.20		3*
	3.70								0.00	1.70	1.76	0.97		7*
								1.51	0.00	1.82	1.43	1.27		10
					0.83			4.14	0.00	1.97	1.97	1.00		12
									0.00	0.79	0.74	1.06	6.8	14
4.11								4.11	0.00	1.83	1.97	0.93	7.4	15
							1.39		0.00	1.36	1.31	1.04	7.2	18
								2.47	0.00	1.27	1.18	1.07	6.8	19
								3.68	0.00	1.85	1.75	1.06	7.2	20
						1.20			0.00	0.57	0.57	1.00	7.3	21
								4.01	0.00	2.15	1.91	1.13	7.5	22
					1.75				0.00	1.89	1.67	1.13	7.4	24
									2.00	1.95	1.03	5.2		
									2.01	1.97	1.02	5.2		

solutions obtained were not sufficiently clear for satisfactory polariscope readings and were therefore discarded.

On the whole our results would appear to confirm the results of neither of the above groups of investigators, for while the majority of our findings were negative in character and were thus in accord with the results of Stiven and Reid, nine experiments gave unmistakable evidence of the existence of a mutarotation, which however, could scarcely be ascribed to the transitory formation of γ -glucose, as, while in five experiments an

upward rotation was noted, in four others carried on under identical conditions, a downward rotation of considerably greater magnitude was found.

From our experience with the problem it would seem that in order to prove the transitory formation of γ -glucose under the conditions of these experiments it will be necessary to resort to experimental technique other than that hitherto employed.

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ADENOSIN HEXOSIDE FROM YEAST.

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In the year 1912, Mandel and Dunham¹ discovered among the extractives of a commercial yeast product "xymin," a hexose-purine complex. The basic component of the substance was identified by them as adenine. The structure of the second component, the hexose, remained unknown. Mandel and Dunham prepared the phenylosazone of the sugar and on the basis of the melting point of the osazone suggested that it might be gulosazone.

Today the study of the naturally occurring hexose nucleosides is of great importance. The nature of the sugar entering into the structure of the thymus nucleic acid still remains unknown. It is not excluded that some of the naturally occurring purine hexosides may have the same relation to thymus nucleic acid as the naturally occurring purine pentosides have to plant nucleic acid. Vernine was discovered by Schulze² long before it was realized that it was a part of the molecule of plant nucleic acid.

In the course of the work on the chemistry of yeast undertaken for a different purpose, the purine hexoside of Mandel and Dunham was isolated. It was decided to make use of this material for the study of the nature of the sugar.

The results thus far obtained seem to justify the conclusion that the sugar possesses unusual peculiarities and seems to differ from other known hexoses. Unfortunately, the hexoside can be isolated only in very small quantities and it will require considerable time before enough material is collected to enable a complete solution of the problem. The evidence obtained to-day indicates that the sugar is a ketohexose, but apparently not a

¹ Mandel, J. A., and Dunham, E. K., *J. Biol. Chem.*, 1912, xi, 85.

² Schulze, E., *Z. physiol. Chem.*, 1910, lxvi, 128.

common 2-ketohexose. The ketonic nature of the sugar was made evident by its behavior towards an aqueous solution of bromine. It is known that aldo sugars are oxidized by bromine water while ketoses are not attacked by this reagent. On standing for 3 days in the presence of a considerable excess of bromine the reducing power of the sugar solution remained unchanged. On oxidation with nitric acid it was impossible to isolate any one of the tetroxyadipic acids obtainable on the oxidation of the known hexoses.

Levene and La Forge³ have shown that the character of the mutarotation of the phenylosazones of a sugar may serve as a directing point in the investigation of the configuration of a sugar. Thus phenylglucosazone is levo-rotatory and on standing, the magnitude of the rotation falls progressively. The same course of mutarotation is also characteristic for altrosazone. The two osazones, however, differ in their melting points. Galactosazone is dextro-rotatory, the magnitude of rotation decreasing with the progress of mutarotation. Gulosazone has a slight initial rotation to the right which increases on standing. These conditions hold for the *d* series.

The phenylosazone of the new sugar resembles considerably that of gulosazone in the character of its mutarotation, the initial rotation being practically 0° and the equilibrium slightly to the right. The melting point was found at 165°C. (corrected) and the decomposition point at 208°C. Thus the mutarotation was not identical but resembled that of gulosazone; but the melting points of both were nearly the same. A marked difference was noted in the decomposition points. Whereas gulosazone was found to decompose at 180°C. the new osazone decomposed at 208°C. Also, in its solubility in water and in alcohol, the new osazone differed from gulosazone, being less soluble than the latter. Finally, the melting point of the two mixed showed a depression from the melting point of each. The mixed substance contracted at 130°C., melted at 140°C., and decomposed at 170°C. More conclusive were the differences brought out in the behavior of the two *p*-bromophenylosazones. The value of the equilibrium rotation of the new *p*-bromophenylosazone was lower than

³ Levene, P. A., and La Forge, F. B., *J. Biol. Chem.*, 1915, **xx**, 429.

that of the corresponding derivative of gulose. The equilibrium rotation of the *p*-bromophenylgulosazone in concentration of 0.100 gm. in 5 cc. of pyridine and alcohol was $+0.32^\circ$, that of the new sugar was $+0.12^\circ$. The melting point of this osazone of the new sugar was 213°C ., that of gulose 186° , with decomposition at 190°C . The *p*-bromophenylhydrazone of the new sugar was also prepared, but the corresponding derivative of sorbose is not known and for the present this derivative cannot be made use of for comparing the two sugars.

Mention may also be made of the reducing power of the product of hydrolysis of the hexoside. A systematic study of the maximum yield of reducing substance, depending on the time of hydrolysis, was made. It was found that the maximum yield was obtained when the hydrolysis was carried out by boiling for 30 minutes, with a reflux condenser, a solution containing 1.0 gm. of the hexoside to each 50 cc. of 1 per cent sulfuric acid. Taking glucose as a standard of reduction, 1 gm. of adenine hexoside should yield 0.6 gm. of sugar. The hydrolysis product, however, showed a reducing power of 0.180 gm. per gm. of the hexoside. A similar yield of reducing substance was obtained when the cleavage was carried out in methyl alcohol by passing through it a stream of hydrogen chloride. The optical rotation of a 1 per cent solution calculated on the basis of the actual reduction, namely on the basis 0.180 gm. per gm. of hexoside, is $+0.69^\circ$; calculated on the basis of the sugar content in the hexoside, namely 0.6 gm. per gm. of hexoside, the optical rotation of a 1 per cent solution of the sugar is $+0.20^\circ$. Neither one of these values agrees with that known for sorbose. Furthermore, on the basis of the rotation of the osazones, the sugar should belong to the *d* series but *d*-sorbose (nomenclature of Rosanoff) rotates to the left, whereas the new sugar is dextro-rotatory.

Thus the new sugar does not seem to be identical with any one of the known 2-ketohexoses. Further work on the sugar is in progress.

EXPERIMENTAL PART.

Brewer's yeast, pressed and washed in lots of 25 pounds, is extracted with 20 liters of alcohol. The extract is concentrated to 1,000 or 1,500 cc. 4 volumes of 95 per cent alcohol are then

added. A precipitate is formed which is removed by filtration and the filtrate is concentrated to 300 cc. 2 liters of 98 per cent alcohol are now added. The precipitate formed after this operation is removed by filtration and the filtrate again concentrated to 300 cc. On standing, a white crystalline precipitate settles out of this solution which consists of leucine. To the mother liquor about 2 liters of acetone are added. Again an amorphous precipitate is formed which is removed by filtration and the mother liquor is concentrated to a small volume. On standing, a white deposit settles out, which consists of microscopic needles. It requires several days before crystallization is completed. For purification the substance is recrystallized out of dilute methyl alcohol. The pure substance melted at and analyzed as follows:

0.1088 gm. dry substance: 0.1768 gm. CO_2 and 0.0494 gm. H_2O .

0.1982 " substance required (Kjeldahl) 33.10 cc. 0.1 N acid.

$\text{C}_{11}\text{H}_{15}\text{N}_5\text{O}_6$. Calculated. C 44.41, H 5.09, N 23.57.

Found. " 44.31, " 5.08, " 23.38.

The optical rotation of the substance dissolved in a 5 per cent solution of sodium hydroxide was as follows:

$$[\alpha]_D^{20} = \frac{-0.08^\circ \times 100}{1 \times 1} = -8^\circ$$

Cleavage of the Purine Hexoside.

The cleavage was carried out with acids as catalyst either in methyl alcohol or in water. For identification of the base the alcohololysis was found the more convenient one.

5.0 gm. of the hexoside were suspended in 50.0 cc. of methyl alcohol and dry hydrogen chloride gas was passed for 10 minutes. The hexoside rapidly dissolved and soon a crystalline precipitate of adenine hydrochloride settled out. The reaction product was allowed to stand overnight and the precipitate filtered and dried. The weight of the dried crude adenine hydrochloride was 2.7 gm. (theoretical yield = 3.0 gm.). Once recrystallized out of water and air-dried, it analyzed as follows:

0.0986 gm.: 0.1208 gm. CO_2 and 0.0344 gm. H_2O .

0.1000 " required for neutralization (Kjeldahl) 27.80 cc. 0.1 N acid.

$\text{C}_6\text{H}_5\text{N}_5\text{HCl} + \frac{1}{2} \text{H}_2\text{O}$. Calculated. C 33.24, H 3.90, N 38.78.

Found. " 33.40, " 3.89, " 38.92.

The filtrate from adenine hydrochloride was used for an oxidation experiment. For this purpose it was diluted with water to the volume of 300 cc., freed from hydrogen chloride in the usual way, and the solution concentrated to 25 cc. An equal volume of nitric acid was added and the solution allowed to stand overnight at room temperature. In several experiments the conditions for further oxidation were varied as to duration of time of heating prior to the concentration of the solution. In none of the experiments was the attempt to prepare a crystalline oxidation product successful. The aqueous solution remains optically active, but it failed to form either an acid potassium salt or a crystalline calcium or copper salt. On the basis of these experiments it was suspected that the sugar was a ketohexose.

Aqueous Hydrolysis of the Hexoside.

3.0 gm. of the hexoside were taken up in 100 cc. of 1 per cent sulfuric acid and heated with reflux over a free flame for 30 minutes. Preliminary experiments had shown that these were the optimal conditions. The colorless reaction product was neutralized with barium carbonate and to the filtrate a slight excess of silver sulfate was added to precipitate the base. The filtrate from the purine silver precipitate was freed from silver and sulfuric acid in the usual way. The total solution had a reducing power equivalent to 0.540 gm. of glucose. Had the sugar of the hexoside been glucose, the reducing power should have been equivalent to 1.80 gm. of the sugar. Not knowing the reducing power of the sugar, it is not possible to determine its specific rotation from the value of the rotation of the solution on hand. However, if only 0.540 gm. were actually present in the solution, the specific rotation of the sugar would be as follows:

$$[\alpha]_D^{20} = \frac{+0.75^\circ \times 100}{2 \times 0.54} = +69.4^\circ$$

Phenylosazone of the Sugar.—3.0 gm. of the hexoside were hydrolyzed by boiling with reflux condenser for 30 minutes. From this experiment a solution was obtained with a reducing power equivalent to 0.550 gm. of glucose. The volume was 150 cc.; to this solution, a solution of 2.0 gm. of colorless phenylhydrazine in glacial acetic acid was added and the solution placed

on a boiling water bath. After 10 minutes a crystalline osazone settled out. The flask was kept on the water bath 1 hour and the final reaction product allowed to stand at 0°C. overnight. The following day the osazone was filtered and suspended in a mixture of alcohol and ether. Part remained insoluble and consisted of microscopic needles of a light orange-yellow color. From the alcoholic ethereal mother liquor on standing, a second crop of crystals appeared. The substance was dried under reduced pressure at the temperature of water vapor. It melted at 164°C. (corrected) and decomposed at 208°C. (corrected). It analyzed as follows:

0.1000 gm. substance: 13.4 cc. nitrogen gas at $t = 20^{\circ}\text{C.}$ and $p = 759.8$ mm.

$\text{C}_{18}\text{H}_{22}\text{N}_4\text{O}_4$. Calculated. N 15.64.
Found. " 15.61.

The optical rotation of the substance in pyridine alcohol solution was

$$\begin{array}{cc} \text{Initial.} & \text{Equilibrium.} \\ [\alpha]_D^{17} = \frac{+0.06^{\circ} \times 100}{1 \times 1} = +6^{\circ} & [\alpha]_D^{17} = \frac{+0.07^{\circ} \times 100}{1 \times 1} = +7^{\circ} \end{array}$$

A sample of the substance 9 months old was crystallized out of alcohol and ether. The substance had the normal appearance of the freshly prepared osazone, but it melted at 194°C. (corrected) and decomposed at 212°C. Also, a second sample of the same age, but not recrystallized, melted at 200° and decomposed at 214 (corrected). Whether this behavior is a peculiarity of this particular osazone or is a common property of all phenylosazones is at present not known. Since the present osazone had some similarities with gulosazone, the properties of the two were compared. It was found that the freshly prepared gulosazone was considerably more soluble in boiling water and in an alcohol-ether mixture. It was mentioned above that when a solution of the new sugar is warmed on the water bath with phenylhydrazine acetate the osazone begins to crystallize after 10 minutes. From a solution of gulose and phenylhydrazine acetate, heated on the water bath 1 hour, the osazone settles out only after standing overnight at 0°C. The phenylgulosazone melts approximately at the same temperature as the phenylosazone of the new sugar,

namely at 160°C., but its decomposition point is much lower; namely, at 185°C. The melting point of a mixture of the two osazones is lower than that of either one. The mixture contracts at 130°C., melts at 140°C., and decomposes at 175°C. The optical rotation of the phenylgulosazone is slightly higher than that of the new sugar; namely,

Initial.	Equilibrium.
$[\alpha]_D^{20} = 0.00^\circ$	$[\alpha]_D^{20} = +16^\circ$

p-Bromophenylosazone.—The non-identity of the new sugar with either gulose, idose, or sorbose is most convincingly shown by the differences in the properties of the two *p*-bromophenylosazones.

A solution containing 0.825 gm. of the new sugar was warmed on the water bath with a solution of glacial acetic acid containing 2.5 gm. of *p*-bromophenylhydrazine. An osazone began crystallizing very soon. The solution was kept on the water bath 1 hour and was then allowed to stand overnight at 0°C. The crystalline osazone was mixed with droplets of a brown oil. It was therefore carefully washed with glacial acetic acid until most of the oil was removed, and then with alcohol and ether. The osazone consisted of bright yellow needles. This substance (not recrystallized) contracted at 217°C. and melted at 226° (corrected). The melting point of *p*-bromophenylgulosazone was 186°C. (corrected). Neuberg and Heymann⁴ report for the substance the melting point of 181°C.

The *p*-bromophenylosazone of the new sugar analyzed as follows:

0.1000 gm. substance: 1.4 cc. of nitrogen gas at $t = 23^\circ\text{C.}$ and $p = 750.2$ mm.

$\text{C}_{13}\text{H}_2\text{ON}_4\text{O}_{12}$.	Calculated.	N 10.85.
	Found.	" 10.70.

The rotations of the two *p*-bromophenylosazones in pyridine alcohol solution were as follows:

New sugar.		Gulose.	
Initial.	Equilibrium.	Initial.	Equilibrium.
$-\frac{0.02 \times 100}{1 \times 1} = -2^\circ$	$+\frac{0.04 \times 100}{1 \times 1} = +4^\circ$	$[\alpha]_D^{20} = 0.00^\circ$	$+\frac{0.16 \times 100}{1 \times 1} = +16^\circ$

⁴ Neuberg, C., and Heymann, F., *Beitr. chem. Physiol. u. Path.*, 1902, ii, 201.

p-Bromophenylhydrazone.—5.0 gm. of the hexoside were hydrolyzed in a solution of 150 cc. of 1 per cent sulfuric acid. The sugar solution was concentrated to a thick syrup. This was taken up in 98.5 per cent alcohol to remove all impurities and concentrated under reduced pressure to a thick syrup. The operation was once repeated. The residue was then taken up in about 10 cc. of 98.5 per cent alcohol and placed on a water bath. To the solution 1.0 gm. of *p*-bromophenylhydrazine was added and the solution kept on the water bath until the hydrazone began to crystallize. Ether was then added to complete crystallization. The hydrazone was then filtered and recrystallized from 98.5 per cent alcohol. It melted at 134–137°C. (corrected) and analyzed as follows:

0.014 gm. substance: 8.0 cc. of nitrogen gas at 24°C. and $p = 755.1$.

$C_{12}H_{17}N_2O_5$ Br. Calculated. N 8.02.

Found. " 8.01.

The specific rotation of the substance in methyl alcohol was as follows:

$$[\alpha]_D^{20} = \frac{+ 0.38^\circ \times 100}{1 \times 1} = + 38^\circ$$

Oxidation with Bromine.

5.0 gm. of the hexoside were hydrolyzed in 150 cc. of 1 per cent sulfuric acid. The base was removed in the usual way. The base-free solution had a reducing power equivalent to 0.894 gm. of glucose. The solution was made up to 25.0 cc. To the solution, in the course of the following 48 hours, an additional 2.0 gm. of bromine were added, and in the course of the following 48 hours, another 2.0 gm. of bromine were added. At the end of 72 hours, the bromine was removed in the usual way and the reducing power of the solution was then equivalent to 0.825 gm. of glucose. Thus it is evident that the sugar was not oxidized by means of bromine.

ON WALDEN INVERSION.

PAPER I.

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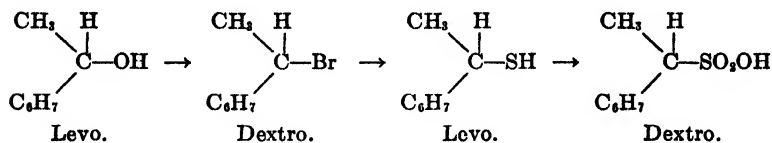
The process of Walden inversion still remains unexplained. A great deal of experimental material has already accumulated and many theories have been advanced. Yet it is generally agreed that, at the present date, there exists no way to discover the exact moment when a Walden inversion occurred. As a rule, the inversion is a resultant of several reactions and the actual problem is to find a way to discover that reaction in which the inversion occurs. In order to facilitate the solution of this problem, the present work was undertaken. It aims to establish the effect on the direction of the optical rotation of a change in the polarity of one of the radicles attached to the asymmetric carbon atom when the change in polarity is brought about without substitution. *A priori*, it is possible that in an asymmetric

substance of the type $\begin{array}{c} R_1 \\ | \\ R_2-C-X \\ | \\ R_3 \end{array}$, the change of rotation with the

change of polarity of the group X may depend on either the structure or the polarities of the groups R_1 , R_2 , and R_3 . This last consideration necessitates the investigation of a large group of substances. As far as the present writers could ascertain, only one instance of a reaction of this type has been described; namely, the oxidation of the optically active amyl alcohol

$\begin{array}{c} CH_3 \\ \diagdown \\ CH-CH_2OH \\ \diagup \\ CH_3 \end{array}$ to the corresponding acid. This reaction is ac-

accompanied with a change of direction of rotation. The present communication deals with the oxidation of mercaptans into sulfonic acids. The particular substance reported here is derived from methylhexyl carbinol. The set of reactions which led to the sulfonic acid and the corresponding changes in rotation are as follows:



Thus it is seen that in this instance, the change of the polarity of one group, brought about without substitution, results in a change of the direction of rotation. This work will be continued on a large number of substances, varying the radicles R_1 , R_2 , and R_3 , and will be extended also to phosphines, arsines, and amines.

There is a second difficulty in the way of a successful solution of the Walden inversion. It is the following. Depending on the character of the reagent, a substitution of one group by another, for instance of a hydroxyl by halogen, may result in substances having opposite direction of rotation. The action of each reagent, for instance of SOCl_2 , is not uniform. It is important to ascertain to what extent the structure of the radicles R_1 , R_2 , and R_3 , determines the outcome of the reaction. Frequently chlorination with SOCl_2 leads to a substance rotating in a direction opposite to that obtained on chlorination with other reagents. Simple aliphatic secondary alcohols have not yet been chlorinated by means of thionyl chloride. Chlorination of these alcohols by other reagents leads to a change of direction of rotation. Active methylhexyl carbinol was acted upon by thionyl chloride and it was found that both the final product as well as the intermediary product R-SOCl rotated in a direction opposite to that of the original alcohol. Thus, in the case of methylhexyl carbinol, substitution by a halogen group always leads to a change of rotation regardless of the reagent employed.

EXPERIMENTAL PART.

d-2-Bromo-octane.

The levo-rotatory secondary octyl alcohol used in this experiment was prepared from the racemic substance according to the method of Pickard and Kenyon.¹ 60 gm. of the alcohol ($[\alpha]_D = -7.51^\circ$) were distilled slowly with 240 gm. of hydrobromic acid (B.P. $124^\circ\text{C}.$). The distillate divided into two layers which were separated by means of a separatory funnel. The aqueous layer was diluted with water and extracted with ether. This extract was combined with the bulk of the bromide and washed with water until the washings showed no presence of hydrobromic acid. After drying with sodium sulfate, the substance was fractionated under about 16 mm. pressure.

	B P.	Weight.	$[\alpha]_D$
	$^\circ\text{C}$	gm.	
F I	Up to 65	20	$+10.71^\circ$ ($C = 100, l = 1$)
F II	65	24	$+13.42^\circ$ ($" = 100, " = 1$)
F III	65-70	16	$+14.56^\circ$ ($" = 100, " = 1$)

F II..... 0.0992 gm. substance: 0.936 AgBr.

F III..... 0.1240 " " : 0.1212 AgBr.

$\text{C}_8\text{H}_{17}\text{Br}$. Calculated. Br. 41.41

Found (F II). " 40.15.

" (F III). " 41.59.

l-2-Mercapto-octane.

20 gm. of *d*-bromo-octane, described in the above experiment (F II), were gradually poured into 60 cc. of 30 per cent solution of alcoholic potassium hydrogen sulfide. The mixture was allowed to stand for 1 hour at room temperature which was followed by heating for 1 hour on a steam bath under a return condenser. On addition of about 3 volumes of water, an oil separated, which was extracted with ether, dried over sodium sulfate, and fractionated.

¹ Pickard, R. H., and Kenyon, J., *J. Chem. Soc.*, 1907, xci, 2058. Norris, J. F., *Am. Chem. J.*, 1907, xxxviii, 627.

	B.P.	Weight.	$[\alpha]_D$
	°C.	gm.	
F I	63-64	7	+8.64° (C = 100, l = 1)
F II	64-64	5	+9.30° (" = 100, " = 1)

F I.....0.2060 gm. substance: 0.3036 gm. BaSO₄.

F II.....0.2058 " " : 0.2896 " "

C₈H₁₈O₃S. Calculated. S 21.81.

Found (F I). " 20.31.

" (F II). " 19.33.

d-Octane-2-Sulfonic Acid.

12 gm. of *l*-mercapto-octane were gradually added to 25 cc. of concentrated nitric acid (sp. gr. 1.42). The flask containing the nitric acid was provided with a return condenser and a dropping funnel. The mercaptan was added through the latter, with occasional shaking. After all the mercaptan had been added and the reaction had somewhat subsided, the reaction mixture was poured into an evaporating dish and the nitric acid was removed by evaporation on a steam bath. A little water was added from time to time. When practically all the nitric acid was gone, the residue was diluted with about 300 cc. of water and an excess of barium carbonate was added. The solution was filtered hot. On cooling, barium salt of the sulfonic acid separated. The residue was extracted several times with hot water; the filtrates were combined and concentrated to a small volume. The barium salt obtained here was combined with the first crop. The salt was washed with a little 1:1 alcohol and ether, then recrystallized, and finally washed again with alcohol and ether. For analysis, the salt was dried at 100°C. in vacuum.

0.1928 gm. substance: 0.1690 BaSO₄.

C₁₈H₃₄O₆SBa. Calculated. S 12.23, Ba 26.25.

Found. " 12.04, " 25.88.

1 gm. of the salt was suspended in a little water and 2 mols of 1.5 N H₂SO₄ were added. The mixture was shaken for 1 hour at room temperature on a shaking machine. The barium sulfate was then filtered off, the filtrate made up to 15 cc., and rotation

determined. Since the barium salt contains 26.25 per cent barium, 1 gm. of the salt corresponds to 0.7375 gm. of acid.

$$[\alpha]_D = \frac{+0.25^\circ \times 100}{2 \times 4.916} = +2.54^\circ$$

Action of Thionyl Chloride on l-Octyl Alcohol.

20 gm. of *l*-octyl alcohol ($\alpha = -7.50^\circ$) were added to 60 gm. of thionyl chloride with cooling. The mixture was then allowed to stand for 1 hour at room temperature, whereupon it was fractionated under a pressure of about 16 mm.

The fraction boiling up to 50°C. was discarded as it was found to consist mainly of unchanged thionyl chloride. The second fraction, boiling between 50° and 79°C. , yielded 12 gm. and showed a specific rotation of $[\alpha]_D = \frac{+14.75^\circ \times 100}{1 \times 100} = +14.75^\circ$.

Analysis showed it to contain 8.5 per cent of ionizable chlorine, 23.56 per cent of total chlorine, and 3.06 per cent of sulfur. Fraction II, distilled at 79°C. , yielded 8 gm. and showed an optical rotation of $[\alpha]_D = \frac{+26.68^\circ \times 100}{1 \times 100} = +26.68^\circ$. Analysis showed it to contain only a trace of ionizable chlorine and no sulfur.

F I 0.2210 gm. substance (Volhard): 5.30 cc. 0.1 N AgNO_3 ,
0.1055 " " (total Cl): 0.1006 gm. AgCl .

0.1908 " " : 0.0426 gm. BaSO_4 .

F II 0.1396 " " : 0.1362 gm. AgCl .

$\text{C}_8\text{H}_{17}\text{Cl}$. Calculated. Cl, 23.90, S 0.

$\text{C}_8\text{H}_{17}\text{SOCl}$. " " 16.71, " 15.06.

Found (F I). Ionized Cl 8.5, total Cl 23.56, S 3.06.

" (F II). " " 24.13.

d-Dioctyl Sulfite.

35 gm. of *l*-octyl alcohol ($[\alpha]_D = -7.15^\circ$) were mixed with 105 gm. of thionyl chloride. The mixture was heated on a steam bath for 10 minutes, whereupon it was poured into cracked ice. When all the thionyl chloride had decomposed, the oil was extracted with ether, washed with dilute NaOH , then with water, and finally dried over Na_2SO_4 . The ether was then removed and the residue fractionated under a pressure of about 16 mm. Two fractions were obtained.

	B.P.	Weight.	$[\alpha]_D$
	$^{\circ}\text{C.}$	gm.	
F I	55-183	7	+9.60° (C = 100, l = 1)
F II	183-187	20	+14.54° (" = 100, " = 1)

Fraction II was redistilled.

	B.P.	Weight.	$[\alpha]_D$
	$^{\circ}\text{C.}$	gm.	
FII ₁	171-178	5	Not determined.
FII ₂	178-179	13	+14.89° (C = 100, l = 1)

F II₂ analyzed as follows:

0.1250 gm. substance: 0.2850 gm. CO₂ and 0.1269 gm. H₂O.

0.2254 " " : 0.1738 " BaSO₄.

C₁₂H₁₄O₈S. Calculated. C 62.74, H 11.20, S 10.45.

Found. " 62.75, " 11.25, " 10.50.

EXPERIMENTAL STUDIES ON PALLADIUM ELECTRODES.

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INTRODUCTION.

The need of a careful scrutiny of the standards and methods employed in the determination of hydrogen ion concentrations by means of the hydrogen electrode is becoming more and more apparent. Despite the ease and accuracy with which colorimetric determinations of pH values may be made on some biological fluids, the electrometric method must still be regarded as standard and the present well merited vogue of pH measurements is such that no detail of the standard procedure is too slight to warrant close study.

In describing the preparation of hydrogen electrodes, many investigators, particularly the authors of texts, have indicated that platinum, palladium, and iridium may be used interchangeably as the medium for the reversible reaction between molecular and ionic hydrogen (1, 2). Platinum has been chiefly employed by the majority of investigators. Iridium and palladium have had but limited use, but the latter has been frequently recommended because of the ease with which it may be removed from old electrodes by anodic electrolysis in hydrochloric acid. This has been regarded as an important advantage, particularly in biological investigations where measurements in protein solutions necessitate the frequent use of newly prepared electrodes. Indeed, it is probable that the biological importance of pH determinations is chiefly responsible for the extent to which palladium has been recommended. While palladium-black electrodes have undoubtedly been used with success by some investigators, verbal communications from a number of others have led to the conclusion that this metal has more often been discarded after a few trials

in favor of the less erratic platinum-black. During the course of attempts to find in palladium a satisfactory substitute for platinum, similar results were obtained in this laboratory and it soon became apparent that an investigation of palladium electrodes and their mechanism as compared with that of platinum was highly advisable. In this investigation, of which the results are recorded below, it was attempted to define the optimum conditions for the preparation of palladium-black electrodes and to indicate a theoretical basis for the differences observed between palladium and platinum.

Method.

Apparatus and Reagents.

All measurements were made with a Leeds and Northrup Type K potentiometer and a sensitive galvanometer. The hydrogen electrode vessels used were of the Clark type as modified by Cullen (3). The small thermometers used in these cells were calibrated against a U. S. Bureau of Standards thermometer. Electrolytic hydrogen obtained from a cylinder was used after being passed successively through a train of wash bottles containing concentrated NaOH, sodium pyrogallate, and water.

The determinations were carried on at room temperature and were corrected for the same in all cases where temperature played an appreciable part; *i.e.*, the correction of barometric readings, the temperature coefficients of Weston and calomel cell, etc. As will be noted below, the effect of temperature variation on the actual pH of the 0.1 N HCl used as a standard amounts to only 0.005 pH for a total change of 18°C. Assuming, therefore, that the necessary corrections had been made, the use of a thermostat was unnecessary. Our purpose being to investigate the electrode *per se*, standard 0.1 N HCl was used for most of the measurements. The acid was standardized through a 0.2 N NaOH solution against repeatedly crystallized acid potassium phthalate and was also checked against 0.1 N HCl prepared by the constant boiling method of Hulett and Bonner (4). The pH of this solution, according to the activity data of Noyes and Ellis (5) was taken as 1.085 at 20°C. Since their data give a value of 1.090 at 38°C. and the temperature curve may be safely assumed to be nearly a straight line, it is evident that the effect of ordinary temperature varia-

tions is negligible. However, in order not to subject the Weston and the calomel cells to undue temperature changes, care was taken to keep the room temperature as nearly constant as possible. In those cases in which it was desired to use buffer solutions near neutrality, KH_2PO_4 - NaOH solutions were made up from carefully standardized materials.

The nearness with which these figures of Noyes and Ellis are approximated is, of course, partially dependent upon the values assigned to the Weston and the calomel cells. The Weston cell used as standard was compared with another cell tested by the Bureau of Standards. The saturated calomel cells used were made from mercury and calomel furnished by the Eppley laboratories. The KCl was four times recrystallized from c. p. material. These cells proved very satisfactory. No "drifting" was in evidence and several cells showed, at different times, a maximum variation among themselves of less than 0.1 millivolt. The values used for these cells were interpolated from those given by Clark (1) in appendix Table A. Using these values and correcting to the nearest 0.5°C ., the data listed below were obtained. Final results are expressed only in terms of pH, the intermediate data being omitted for the sake of brevity.

Detailed Procedure.

Preliminary experiments indicated the necessity of about 10 to 15 minutes shaking of electrode vessels containing solution and electrode in contact with hydrogen before equilibrium was attained. For this reason a standardized interval of 15 minutes shaking was adopted for each reading. The procedure was as follows: The electrode vessel was first rinsed and filled with the standard solution and the stopper containing the electrode under examination placed in position in such a way as to leave the vessel completely filled with liquid. A portion of the solution was then expelled by means of hydrogen and the vessel was rocked for 15 minutes.

Immediately after the 15 minute period of shaking, the hydrogen inlet was closed, the connection into the salt bridge opened and the reading taken at once in order to minimize any error due to contact potential between the 0.1 N HCl and the saturated KCl . Particular care was taken to effect this junction in the same way in each case and to use the same vessel throughout each principal series of determinations. After each reading, the vessel was rinsed and refilled with a new portion of the standard solution. By thus maintaining a definitely standardized schedule for each determination, it was possible to interpret the data given below in terms of the total time required for each electrode to give equilibrium values in the 0.1 N HCl .

Each successive pH value given represents a 15 minute period plus the few minutes required to empty and refill the electrode vessel. Only the final pH values are recorded.

The small platinum electrodes which fit the Clark-Cullen vessel were employed. These electrodes were of practically constant area throughout; no variations in current density were employed. The procedure used in plating palladium-black upon the platinum was as follows:

The principal palladium solution used contained 3 per cent PdCl_2 . To this were added 2 cc. of concentrated HCl per 140 cc. of solution. These concentrations were chosen in order to duplicate as nearly as possible the concentrations which had given best results in the hands of other investigators. A few preliminary trials showed that more concentrated solutions or more alkaline ones were impractical because of their tendency to deposit basic salts and, further, because electrodes made from freshly prepared solutions of higher concentration showed no superiority over those made from the 3 per cent solution. The question of alkalinity will be considered later. The palladium chloride used was a c.p. product which analyzed 97.86 per cent PdCl_2 by the dimethylglyoxime method of Wunder and Thüringer (6).

The cleaned platinum electrodes were rubbed with very fine emery powder for the purpose of removing any undissolved portion of previous deposits, washed, and placed for several hours in hot chromic acid cleaning solution. They were then washed with distilled water, electrolyzed cathodically in 10 per cent H_2SO_4 , washed again, and electrolyzed in the PdCl_2 solution. For both the preliminary electrolysis in 10 per cent H_2SO_4 and the plating in PdCl_2 , two dry cells in series, giving a total of nearly 2.5 volts, were used. The electrolysis in 10 per cent H_2SO_4 was chiefly used as a test for perfect cleanliness, uniform evolution of hydrogen from the entire surface of the electrode being required. In the actual plating, the same distance between anode and cathode was preserved in each case as closely as possible. It was desired to introduce no variations in amount of current passing and in current density except those caused by actual variations in the composition of the plating solutions. After plating, the electrodes were washed in distilled water, and, except as otherwise stated, used at once. In washing, a fairly strong stream of water from the wash bottle was played on the electrode in order that any loose portions of the deposit might be dislodged. Any visual evidence of uneven distribution or any bright spots on the electrode caused its rejection. The presence of bright spots, showing where the deposit had flaked off, was especially common in the heavier platings. Rejected or used electrodes were cleaned by anodic electrolysis in HCl and then put through the above described cleaning process before replating.

The question as to the optimum depth of plating was early brought to the writer's attention by the complete failure of electrodes due to too heavy deposits. Few investigators have emphasized the advisability of using fairly light deposits of any metal

and the almost universal criterion of proper thickness for platinum deposits has also been generally used for palladium. This is probably one cause for much of the lack of success with palladium electrodes. It has been found that if palladium deposits are carried to the point of the rich, velvet black which is by most workers regarded as the sign of a good platinum deposit, it is almost impossible to prevent flaking. The electrode is thus ruined or is, at best, extremely sluggish. In some of the following experiments this is shown by the use of three different depths of plating. The minimum thickness which gave good results was deposited by only 15 second electrolysis under the conditions described. In some series, therefore, the electrodes were plated 15, 30, and 45 seconds. The plating of 15 seconds which was finally adopted as the standard depth, was so thin that its color was only light grayish. The platinum surface was barely covered.

EXPERIMENTAL.

Variations in Acidity.

The data in Table I show the results of increased acidity of the stock palladium solution. Each successive solution used was made by addition of concentrated HCl to a 15 cc. sample of fresh stock solution. With each solution 15, 30, and 45 second platings were made.

The portion of Table I showing the behavior of electrodes made directly from stock PdCl_2 solution was selected as typical from a large amount of such data. The data in the "15 second electrode" column represent good average results obtained from such electrodes and may be taken as indicating the best results that could be consistently obtained with any form of palladium electrode used. The sluggishness of the 30 and 45 second electrodes is plainly evident. The remaining data in Table I show, barring minor erraticisms, the detrimental effect of increased acidity of the plating solution. The very erratic behavior of electrodes made in the most acid solution is probably due to the fact that the poorer quality of the deposit is temporarily offset by the charging of the electrode with hydrogen during plating. This effect will be discussed later in more detail.

Variations in Dilution.

In Table II are shown the results of diluting the stock solution.

It would seem from the above that although dilution of the PdCl_2 solution with a proportionate increase in the time of elec-

TABLE I.

Effect of Increasing Acidity of the Plating Solution. Each Successive pH Represents a 20 Minute Interval and a New Reading on the Same Electrode.

15 sec. plating.	30 sec. plating.	45 sec. plating.
Stock PdCl_2 solution.		
pH	pH	pH
1.040	1.016	1.008
1.068	1.028	1.015
1.073	1.052	1.029
1.079	1.059	1.032
1.082	1.065	1.043
1.080	1.067	1.048
15 cc. stock PdCl_2 + 1 cc. concentrated HCl.		
1.010	1.002	1.003
1.028	1.009	1.007
1.045	1.019	1.012
1.067		1.035
1.072	1.057	1.041
15 cc. stock PdCl_2 + 2 cc. concentrated HCl.		
1.015	1.008	0.96
1.019	1.026	1.014
1.035	1.028	1.052
1.032	1.035	1.036
1.047	1.046	
15 cc. stock PdCl_2 + 3 cc. concentrated HCl.		
1.061	1.003	0.92
1.052	1.029	0.98
1.066	1.034	1.042
1.056	1.033	1.016
1.048	1.028	1.031

trollysis would be expected to yield about the same results, the actual data obtained are more erratic and show less tendency to

reach the equilibrium value than when a more concentrated solution is used. The "15 second" electrodes obtained were extremely thin and were probably incapable of absorbing sufficient hydrogen to give a reliable electrode.

Effect of Acid Immersion.

During the whole of these experiments, it was often observed that when an electrode which had been used for several determinations was allowed to stand overnight in the standard 0.1 N HCl,

TABLE II.

Effect of Dilution of the Plating Solution. Successive Readings Have the Same Significance as in Table I.

15 sec. plating.	30 sec. plating.	45 sec. plating.
1 volume stock PdCl_2 + 1 volume H_2O .		
<i>pH</i>	<i>pH</i>	<i>pH</i>
1.028	1.043	1.027
1.060	1.059	1.030
1.075	1.071	1.043
1.057	1.081	1.052
1.066	1.084	1.067
1 volume stock PdCl_2 + 2 volumes H_2O .		
1.031	1.013	1.005
1.060	1.047	1.041
1.059	1.059	1.059
1.065	1.066	
1.052	1.068	1.065

the next day's readings invariably showed absurdly low values. For example, a reading of practically equilibrium value would be reduced to less than pH 1 in most cases and subsequent readings showed no very marked increase.

A short series of experiments was made to show the effect of immersion in acids of different concentrations. Four electrodes were made up by the "standard" procedure (15 second electrolysis in 3 per cent PdCl_2 , etc.). Each was used until practically equilibrium values were obtained and then placed overnight in the solution indicated. The next readings were taken immediately after thorough washing the next morning, the period of immer-

sion having been the same in all cases. Table III contains the results obtained both before and after immersion.

The effect of acid is plainly evident, even though the electrode which had been placed in 0.1 N HCl yielded a higher value than was frequently found in similar cases. The experiment with the chromic acid solution was included since it is well known that chromic acid cleaning solution has no detrimental effect on platinum electrodes. It is evident from the results on electrode A that after several hours in water, an electrode can temporarily recover in about normal time. The effect of alkali will be discussed later.

TABLE III

Effect of Immersion of Electrode in Acid. Successive Readings at Intervals of 20 Minutes.

Electrode. . .	A	B	C	D
	<i>pH</i>	<i>pH</i>	<i>pH</i>	<i>pH</i>
Original readings.	1.063	1.073	1.066	1.054
	1.072	1.082	1.073	1.059
	1.072	1.079	1.081	1.068
	1.078	1.080	1.077	1.073
	Immersion overnight in distilled water.	0.1 N HCl	Concentrated HCl.	Chromic acid cleaning solution.
Readings after immersion.	1.030	1.020	0.87	0.85
	1.050	1.029	0.98	0.79
	1.067	1.034	0.92	
	1.074	1.038		

On the other hand, various other experiments showed that the best palladium electrodes do suffer deterioration on standing for longer periods in water. 3 days of immersion in water before use not only made the attainment of any particular pH below the equilibrium value require three to four times as long as in the case of a fresh electrode, but actually prevented the attainment of the equilibrium value within any reasonable length of time. As will be shown later, this is undoubtedly due to a slow permanent change, rather than to any temporary effect of the water in removing hydrogen from the electrode. A slight temporary sluggishness, on the other hand, might be accounted for by a loss of the hydrogen with which the electrode had become partially charged during plating and previous use.

To determine whether or not any beneficial effect resulted from further cathodic electrolysis after plating, several comparisons were made. "Standard" electrodes were used, both directly and after periods of electrolysis of varying lengths of time in solutions of acidity varying from 0.1 N to 10 per cent H_2SO_4 . Detailed quotation of results is unnecessary since in no case did either any definite improvement or harm result from this electrolysis on *first* using the electrodes. Evidently the electrode becomes fairly well saturated during plating and any further treatment with acid merely aids in hastening its *final* deterioration.

TABLE IV.

Progress of Deterioration of Electrodes. Successive Readings Have the Same Significance as in Preceding Tables.

1st day.		2nd day.		3rd day.	
Electrode.		Electrode.		Electrode.	
A	B	A	B	A	B
pH	pH	pH	pH	pH	pH
1 035	1 050	1 026	0 995	0 955	1 006
1 060	1 055	1 043	1 033	1 017	1 038
1 064	1 058	1 051	1 034	1 043	1 049
1 074	1 076	1 060	1 045	1 056	
1 072	1 071	1 068	1 057	1 059	1 004
1 074	1 073	1 064	1 066	1 048	0 99
		1 065	1 057	Both electrolyzed in 10 per cent H_2SO_4 for 1 min.	
				1 066	1 062
				1 023	1 037
				1.006	1 000

That cathodic electrolysis following plating does contribute to the final deterioration of an electrode is shown by the data in Table IV. Two "standard" electrodes were made: A was used directly in 0.1 N HCl while B was first electrolyzed for 1 minute as the cathode in 10 per cent H_2SO_4 . Both were then used for 3 successive days and over the 2 intervening nights were kept in distilled water, under hydrogen. After the 3rd day's measurements were made and the deterioration of the electrodes became very marked, both were electrolyzed for 1 minute, each in 10 per cent H_2SO_4 , and immediately used again. The results are highly interesting.

The progressive deterioration is quite easily apparent, particularly in the case of electrode *B*, while the resaturation of both electrodes, on the 3rd day, by electrolysis in 10 per cent H_2SO_4 produced a temporary rise only as long as the effect of the hydrogen lasted. The rapid drop which followed at once clearly shows that the electrodes were no longer capable of acting reversibly.

That this deterioration was at least partially independent of the pH of the solution in which the electrode was used is indicated by two facts: (1) that electrodes immersed 2 or 3 days in water before use are correspondingly sluggish; and (2) that electrodes used in solutions of higher pH than 0.1 *N* HCl during successive days deteriorate, although relatively less rapidly. For example, an electrode used for 3 successive days in a phosphate buffer of pH 6.8 gave as its maximum reading for each day, pH 6.802, 6.783, and 6.772. The bad effects of electrolysis in 10 per cent H_2SO_4 before use were illustrated in a number of cases. For example, two electrodes were prepared exactly as were those of Table IV but were preserved in distilled water and used 1 day after preparation instead of immediately. The results were comparable to those of the 2nd day in Table IV, but the unfavorable effect of the electrolysis of one electrode in 10 per cent H_2SO_4 was somewhat more marked.

Effect of Alkali Immersion.

A series of trials was also made with palladium electrodes in which the effect of immersion in alkali of various concentrations was studied. "Standard" electrodes were prepared as described above and were immersed for varying periods of time in NaOH solutions of 0.1 to 1.3 *N*. The first readings obtained in 0.1 *N* HCl were highly promising—almost equilibrium values were obtained at once—but these figures quickly dropped to values as low as pH 1.0 and below. In all cases, the results obtained were too erratic to be worth quoting. Even values obtained on 0.1 and 0.01 *N* NaOH solutions and on nearly neutral buffer solutions after such immersion, showed the same lack of agreement. Variations in the method of preparation of the electrodes, the use, before and after alkali immersion, of cathodic electrolysis, etc., effected no improvement. Altogether, the net result of these experiments was such as to throw considerable doubt on any determinations of high pH values with palladium electrodes.

In this connection the results obtained by Hammett (7) in the course of some studies on platinum-black electrodes are of decided interest. He found in the case of platinum-black electrodes which had had considerable exposure to hydrogen, a high degree of sensitivity towards minute amounts of oxygen when used in solutions of high pH. He says in part: "Indeed the limiting factor upon the use of electrodes is the sensitiveness to oxygen which finally becomes so great that no reasonable precautions can give correct results. It is perhaps not realized how rapidly oxygen can diffuse through an unlubricated ground joint even against a slight pressure of hydrogen." That the deterioration of the palladium electrodes used in acid solution in this laboratory is not due to increasing oxygen sensitivity is evidenced by the fact that no improvement was experienced on exposing the electrodes to atmospheric oxygen. This procedure Hammett found effective in reducing the sensitivity of old electrodes. Moreover, the rate and extent of deterioration under acid conditions as shown by data in this paper are far greater than that ascribed by Hammett to oxygen sensitivity under like conditions. However, it seems very probable that such a mechanism is responsible for some of the results obtained in this laboratory on using alkaline solutions.

It should also be noted that this sensitivity towards oxygen may have considerable bearing on the results obtained from pH measurements in solutions containing oxyhemoglobin. While maximum oxygen sensitivity occurs under conditions far more alkaline than those prevailing in the usual biological range, still, even in 0.1 N HCl the effect is perceptible and may well be of importance near neutrality.

One important factor in the use of hydrogen electrodes, particularly of palladium, which probably does not always receive the minute care it deserves, is the matter of contamination of the electrode with mercury. The use of mercury connections in the glass tube into which the electrode is sealed is very common and the frequent insertion and withdrawing of the copper wire lead may easily result in the spilling of small drops of mercury. This matter was first brought forcibly to the writer's attention when using an electrode in which the platinum wire had been imperfectly sealed into the glass. Very small amounts of mercury crept through and amalgamated with the palladium, completely ruining the elec-

trode. In spite of the fact that metals of the platinum groups are not regarded as readily forming amalgams and that the only inorganic treatise available which mentioned palladium amalgams stated that they were only formed slowly and with difficulty, it has been repeatedly noticed that palladium-black electrodes amalgamate almost instantly when brought in contact with mercury. This amalgam, an easily flowing liquid, may be removed by anodic electrolysis in either dilute HCl or H_2SO_4 with the difference that in the former case both mercury and palladium are removed, leaving a clean platinum surface, while in the latter, the mercury is removed as the sulfate leaving an electrode which is blacker than before amalgamation and which will not again amalgamate—at least under the same conditions. When used as a hydrogen electrode in 0.1 N HCl against a calomel cell it gave only about 40 per cent of the voltage which should be obtained from a good electrode.

A recent communication by Bovie and Hughes (8) calls attention to another source of trouble resulting from the introduction of mercury into the hydrogen electrode vessel. They find that the decomposition of mercurous chloride into mercuric chloride and free mercury, results in a gradual diffusion of the former through the salt bridge to the hydrogen electrode vessel. As a result, the electrode is "poisoned" and rendered useless. The authors recommend that the KCl solution of the salt bridge should at times be tested with sodium sulfide solution for the presence of mercury.

Such tests applied to salt bridge solutions used in this laboratory have thus far given negative results. This may be ascribed to the fact that the calomel employed in the standard half-cell already contained a considerable proportion of finely divided metallic mercury which undoubtedly tended to reverse the action referred to above.

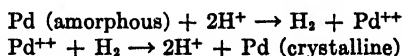
Causes of Electrode Deterioration.

The mechanics of the absorption of hydrogen in palladium have been the subject of much investigation, but only those papers having direct bearing upon the question of hydrogen electrode mechanism will be considered here. Holt, Edgar, and Firth (9), in studying the sorption of hydrogen in palladium, concluded that

an active form of palladium, which is evidently the result of a metastable condition of the metal, is necessary in order that hydrogen be adsorbed.

Andrew and Holt (10) studied further the sorption of hydrogen by palladium and concluded that additional evidence had been found to point to the dimorphic nature of the metal. They also stated that the relative stability of the two forms depends on the temperature. When existing separately, the change from the amorphous to the crystalline form is very slow. That the metal exists in two different states, depending on temperature and mode of treatment, is also indicated by consideration of the heating and cooling curves of palladium in hydrogen.

It seems very probable that the behavior of palladium electrodes recorded in this paper can be most simply explained by the dimorphic nature of the metal emphasized by the investigators quoted above. In the determination of hydrogen ions only the amorphous form of palladium would be expected to yield a thermodynamically reversible electrode. This amorphous form, the more active of the two, exists in metastable equilibrium with the stable crystalline variety and changes to the latter, particularly when the two are in contact. It seems reasonable, therefore, to explain the behavior of palladium electrodes by the supposition that this change to the stable form takes place slowly at all times and quite rapidly under favorable conditions. High hydrogen ion concentrations evidently constitute a favorable condition. Aside from the possibility of some catalytic action of unknown mechanism, it is conceivable that the more active form may well have a solution tension sufficiently higher than the crystalline to permit of an ionic exchange with H^+ .



Such a mechanism would explain the particularly rapid deterioration of palladium electrodes in solutions of high acidity. Experimental evidence dealing with this point will be discussed below.

In this connection, the studies of Sieverts and Peters (11) on the catalytic oxidation of hypophosphorus acid by means of various forms of palladium-black are pertinent. They found that

palladium-black is very active if prepared by reduction with formic acid, hypophosphorus acid, or carbon monoxide, that palladium sponge is less active after heating, and that wire and foil are inactive even if previously saturated cathodically with hydrogen. They also found that the activity of palladium-black, made by electrolytic deposition on platinum or copper, drops very quickly and that this drop is hastened by addition of further quantities of acid. The drop obtained, even without addition of acid, is undoubtedly due to the increasing acidity which results from the formation of the more highly ionized phosphorus and phosphoric acids. The results of Sieverts and Peters throw particular suspicion upon the palladium-black made by electrolytic deposition. It seems probable that this method of preparation results in a product which contains an unusually large proportion of crystalline palladium.

A rough measure of the proportions of the two forms present in various samples of palladium-black was effected by Firth (12) who determined the sorption of hydrogen by various samples in such a way that the results indicated the relative proportions of the two forms. Firth does not seem to have worked with electrolytic palladium-black, but it is interesting to note that the product formed by reducing PdCl_2 with H_2 contains much less of the amorphous phase than does that formed by reducing PdO with H_2 . Here again the action of acid seems to come into play. Altogether, he concludes that the mode of preparation bears a decided influence on the proportion of the two forms present.

In view of the mechanism suggested above, by which amorphous palladium is changed to crystalline, it seemed pertinent to compare the solution tensions of the two forms against a series of three palladium solutions. Pure PdCl_2 solutions were made and their concentration checked by the method of Wunder and Thüringer (6). An electrode of bright, carefully cleaned palladium wire and one of thickly plated palladium-black were used in each of the solutions. Voltages were measured in conjunction with a saturated calomel cell and the solution tension of the metal (K_{Pd}) was calculated by means of the usual formula:

$$E_{\text{Pd}} = 0.0287 \log \frac{C}{K}$$

applying to a divalent metal at 17°C .

These values were calculated on the assumption of complete ionization and no attempt was made to do more than to obtain a comparison of the two forms of palladium. For this reason no steps were taken to determine the cause of the progressive drop in values for K of both forms of palladium, which is of far too great magnitude to be accounted for by the degree of ionization of PdCl_2 , but for which the contact potential between the saturated KCl solution and the dilute PdCl_2 solution is probably responsible. However, the higher solution tension of the less stable form is very evident at all concentrations used. A number of measurements were taken in each case, the above figures for K representing the average of all values obtained. In no case did these values vary by more than 0.3×10 with the same exponent as that of the average value. An older determination of K_{Pd} by Neumann (13) gives the value of 4.0×10^{-36} .

TABLE V.
Solution Tensions of Amorphous and Crystalline Palladium.

C <i>mols per l.</i>	Electrode	E_{Pd} <i>volts</i>	K_{Pd}
0 02805	Crystalline.	0 9512	2.0×10^{-35}
0 02805	Black.	0 8508	6.4×10^{-35}
0 002805	Crystalline.	1 0006	3.9×10^{-35}
0 002805	Black	0 9283	1.3×10^{-35}
0 0002805	Crystalline.	1 0184	9.2×10^{-40}
0 0002805	Black.	0 9776	2.4×10^{-38}

SUMMARY.

1. Palladium electrodes for hydrogen ion determinations are much less reliable than platinum electrodes because of the lack of permanence of the former which results from a more or less rapid change of amorphous to crystalline palladium.

2. This change is positively catalyzed by high hydrogen ion concentrations, the mechanism being probably an electronic exchange facilitated by the higher solution tension of amorphous palladium.

3. The use of palladium electrodes in connection with solutions of high pH gave very erratic results.

4. Optimum conditions for the preparation of palladium electrodes were determined.

5. The relative solution tensions of the two forms of palladium were determined.

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UNIFORMITY IN INVERTASE ACTION.

III. THE STABILITY OF THE ENZYME.*

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Nelson and Hitchcock (1) found that the hydrolysis of sucrose by different preparations of invertase does not follow the same velocity law. Of eight different preparations studied by them, six were uniform, in that their inversion of a 10 per cent sucrose solution at 25°C. and a hydrogen ion concentration of pH 4.5, when plotted as percentage hydrolyzed against time, gave curves which were superimposable when the data were multiplied by a proper constant. They were able to express this uniformity by:

$$t = \frac{1}{n} \left(\frac{\log 100}{(100 - p)} + 0.002642p - 0.00000886p^2 - 0.0000001034p^3 \right)$$

where p is the percentage hydrolyzed at time t and n is a constant of the hydrolysis. These six preparations, Nos. 1, 2, 7, 8, A, and B, gave values of n with an average deviation from the mean less than 0.7 per cent, the experimental error limit set by them, and were termed "normal." The other two preparations, Nos. 3 and 6, gave values of n decreasing progressively beyond the limits of experimental error, and differed in this respect not only from the majority, but also from each other. These preparations were accordingly termed "abnormal."

Nelson and Hollander (2) showed that if they allowed the abnormal preparations of Nelson and Hitchcock to stand, without sucrose being present, at 25°C. and pH 4.9 for 5 hours, which period corresponds to the time required for the hydrolysis of sucrose solution, then these preparations lost activity. The normal preparations, on the other hand, did not lose activity under

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these conditions. In the light of these results, they concluded that the abnormality manifested by these two preparations of Nelson and Hitchcock, was due to the instability of these preparations.

Changing Normal Invertase Preparations to Abnormal by Ferric Oxide Hydrosol.

The fact that Nelson and Hitchcock were able to stabilize their abnormal preparation No. 3 by the addition of normal invertase preparation, which had been previously inactivated by heating, suggests that there might be something in the way of accompanying material, lacking in this abnormal preparation, but present in normal preparations of the enzyme. Accordingly, it was thought advisable to see whether by some method for removing certain parts of accompanying material in normal preparations, the latter would become abnormal. It was obvious from the outset that ordinary methods of preparing the enzyme, which processes involve removal of accompanying material, could not be employed, since these methods usually yield normal preparations. Such well known procedures as duplicate precipitation with alcohol and redispersion with water, treatment with lead acetate, and adsorption on various materials and subsequent elution therefrom, were discarded and a new method was sought.

It was found that the addition of ferric oxide hydrosol, prepared in a manner described elsewhere in this paper, to a normal invertase preparation, No. 8 D, at pH 2.1, produced a precipitate, and upon using a portion of the filtrate for the hydrolysis of a 10 per cent sucrose solution at pH 4.5, this filtrate proved to contain abnormal invertase. Furthermore, it was found that the activity of the filtrate was greater than that of the original solution, when calculated on the basis of the solids still remaining in the solution, and hence showing that some accompanying material had been removed in producing the abnormal from the normal preparation.

In order to decide whether this treatment with ferric oxide hydrosol and the consequent changing of normal preparation to abnormal is at all general, two new preparations, K and M, were prepared by a method quite different from that employed by Nelson and Hitchcock in the case of their No. 8 D. Preparation K was obtained from a sample of yeast that had been allowed to stand for several years, and Preparation M, from a fresh sample

of yeast. Both were obtained by treating the autolyzed yeast juice with alcohol, redissolving the precipitate in water, and repeating the process, as described in a subsequent part of this paper. No. 8 D, a separate portion of No. 8 of Nelson and Hitchcock, removed to a smaller bottle, had been prepared according to the method used by Nelson and Born (3), which, described briefly, consists in further treating the product, obtained by the method

TABLE I.

Abnormality of Invertase Preparations 8 D, M, and K after Treatment with Ferric Oxide Hydrosol.

Hydrolysis with invertase 8 D after treatment with the iron sol.						Hydrolysis with invertase 8 D before treatment with the iron sol.		
Experiment A 1.			Experiment A 2.			Experiment A 7.		
t	R	Relative n.	t	R	Relative n.	t	R	Relative n.
0	25.89		0	25.88		0	25.89	
120	22.07	684	120	22.18	664	120	22.13	672
181	20.24	677	180	20.42	658	180	20.28	675
240	18.50	675	240	18.77	651	240	18.50	675
300	16.84	670	300	17.14	646	300	16.75	676
360	15.24	660	360	15.60	641	360	15.09	675
420	13.78	657	420	14.12	636	420	13.47	675
	-7.43			-7.43			-7.43	
Mean....		672 ± 1.2%	Mean....		649 ± 1.3%	Mean....		675 ± 0.1%

Hydrolysis with invertase K after treatment with the iron sol.						Hydrolysis with invertase K before treatment with the iron sol.		
Experiment A 3.			Experiment A 4.			Experiment A 8.		
t	R	Relative n.	t	R	Relative n.	t	R	Relative n.
0	25.90		0	25.91		0	25.88	
90	19.92	1,441	90	19.95	1,433	90	20.05	1,408
120	18.06	1,435	120	18.09	1,429	120	18.15	1,418
150	16.28	1,428	150	16.32	1,421	150	16.33	1,420
195	13.69	1,424	195	13.74	1,418	180	14.55	1,424
285	9.08	1,411	240	11.36	1,412	210	12.84	1,426
330	7.06	1,406	330	7.12	1,400	285	8.94	1,428
375	5.21	1,400	375	5.20	1,401	330	6.83	1,429
	-7.42		435	3.15	1,390	375	5.04	1,424
				-7.42			-7.44	
Mean....		1,421 ± 0.9%	Mean....		1,413 ± 0.9%	Mean....		1,422 ± 0.4%

TABLE I—*Concluded.*

Hydrolysis with invertase M1 after treatment with the iron sol						Hydrolysis with invertase M before treatment with the iron sol.					
Experiment A 5			Experiment A 6			Experiment A 9.					
<i>t</i>	<i>R</i>	Relative <i>n</i>	<i>t</i>	<i>R</i>	Relative <i>n</i>	<i>t</i>	<i>R</i>	Relative <i>n</i> .			
0	25 89		0	25 89		0	25 89				
60	18 89	2,552	60	18 89	2,552	60	18 99	2,515			
90	15 75	2,521	90	15 74	2,523	90	15 73	2,525			
120	12 86	2,491	120	12 86	2,491	120	12 71	2,525			
150	10 23	2,463	150	10 25	2,459	150	9 91	2,524			
180	7 90	2,433	180	7 91	2,431	180	7 35	2,526			
210	5 83	2,404	210	5 85	2,400	210	5 12	2,522			
240	4 02	2,376	240	4 05	2,370	240	3 11	2,524			
270	2 44	2,349	270	2 47	2,344	270	1 40	2,526			
	-7 42			-7 43			-7 43				
Mean.		2,449 ± 2 4%	Mean.		2,446 ± 2 4%	Mean.		2,523 ± 0 1%			

outlined for Preparations K and M, with lead acetate, then potassium oxalate, and finally dialyzing against running tap water. All these preparations, Nos. 8 D, K, and M, were normal preparations, as an inspection of Table I will show.

All three preparations were treated with ferric oxide hydrosol, according to the procedure given under experimental details, filtered, brought back to pH 4.5, dialyzed, and used for inverting sucrose. The results given in Table I show that they all had been converted now into abnormal preparations. That is, the results obtained from the hydrolysis of a 10 per cent sucrose solution show a progressive decrease in the value of *n*, so great, that the average deviation from the mean is greater than 0.7 per cent, the experimental error limit set by Nelson and Hitchcock for normal preparations.

Changing Abnormal Invertase Preparations to Normal by a Normal Invertase Preparation Which Had been Inactivated by Heating.

Nelson and Hollander showed that the reason why Nelson and Hitchcock were able to change abnormal invertase No. 3 to normal by the addition of an inactivated normal preparation, was due to the stabilizing influence of the latter material upon the abnormal preparation.

TABLE II.

Effect of Added Substances on the Stability of Abnormal Invertase.

Experiment No.	Added substance.	Inactivation period.	Relative hydrolysis velocity constant, n.	Loss in ".
		hrs.		per cent
C 1	None.	0	2, 112	
		4	1, 808	14 4
C 2	10 cc. inactivated normal invertase M.	0	2, 169	
		4	2, 207	0.0
C 4	10 " " " " "	0	2, 203	
		4	2, 204	0.0
C 3	10 cc. inactivated abnormal invertase M.	0	2, 197	
		4	2, 177	0.9
C 5	10 " " " " "	0	2, 192	
		4	2, 168	1 1
D 1	10 cc. 0.01 per cent gelatin solution.	0	2, 291	
		4	2, 293	0.0
D 2	10 cc. 0.01 per cent egg albumen solution.	0	2, 293	
		4 -	2, 298	0.0
D 3	None.	0	2, 552	
		4	2, 370	7.1
D 4	10 cc. 0.01 per cent gelatin solution.	0	2, 703	
		4	2, 701	0 0
D 5	10 cc. 0.01 per cent egg albumen solution.	0	2, 682	
		4	2, 692	0.0 "
E 1	None.	0	2, 130	
		4	1, 962	7 8
E 5	"	0	2, 127	
		4	1, 948	8.4
E 2	0.1 cc. inactivated normal invertase M.	0	2, 135	
		4	2, 025	5 2

TABLE II—Continued.

Experiment No.	Added substance.	Inactivation period.	Relative hydrolysis velocity constant, n .	Loss in n .
		hrs.		per cent
E 6	0.1 cc. inactivated normal invertase M.	0 4	2,135 2,017	5.5
E 3	1.0 " " " " "	0 4	2,143 2,082	2.8
E 7	1.0 " " " " "	0 4	2,138 2,067	3.3
E 4	5.0 " " " " "	0 4	2,150 2,102	2.2
E 8	5.0 " " " " "	0 4	2,153 2,112	1.9

Abnormal invertase M 2.				Abnormal invertase M 1.			
Experiment C 1. pH = 4.7.				Experiment D 3. pH = 4.8.			
I.P. = 0		I.P. = 4 hrs.		I.P. = 0		I.P. = 4 hrs.	
t	Relative n .	t	Relative n .	t	Relative n .	t	Relative n .
60	2,112	60	1,808	60	2,552	60	2,370
90	2,083	90	1,792	90	2,521	90	2,343
150	2,031	150	1,736	150	2,463	150	2,283
First n ...	2,112	First n ..	1,808	First n ..	2,552	First n ..	2,370

Abnormal invertase M 2 + 10 cc. inactive normal invertase M.

Experiment C 2. pH = 4.9.				Experiment C 4. pH = 4.9.			
I.P. = 0		I.P. = 4 hrs.		I.P. = 0		I.P. = 4 hrs.	
t	Relative n .	t	Relative n .	t	Relative n .	t	Relative n .
60	2,177	60	2,210	60	2,205	60	2,213
90	2,179	90	2,212	90	2,208	90	2,202
150	2,151	150	2,200	150	2,197	150	2,197
Mean....	2,169	Mean....	2,207	Mean....	2,203	Mean....	2,204

* Throughout the tables in this article I. P. represents inactivation period.

TABLE II—Continued.

Abnormal invertase M 2 + 10 cc. inactive abnormal invertase M 2.							
Experiment C 3. pH = 4.8.				Experiment C 5. pH = 4.8.			
I.P. = 0		I.P. = 4 hrs.		I.P. = 0		I.P. = 4 hrs.	
<i>t</i>	Relative <i>n.</i>	<i>t</i>	Relative <i>n.</i>	<i>t</i>	Relative <i>n.</i>	<i>t</i>	Relative <i>n.</i>
60	2,197	60	2,177	60	2,192	60	2,168
90	2,188	90	2,162	90	2,174	90	2,150
150	2,144	150	2,115	150	2,130	150	2,109
First <i>n.</i> ...	2,197	First <i>n.</i> ...	2,177	First <i>n.</i> ...	2,192	First <i>n.</i> ...	2,168
Abnormal invertase M 2 + 10 cc. 0.01 per cent gelatin solution.				Abnormal invertase M 1 + 10 cc. 0.01 per cent gelatin solution.			
Experiment D 1. pH = 4.8.				Experiment D 4. pH = 4.9.			
I.P. = 0		I.P. = 4 hrs.		I.P. = 0		I.P. = 4 hrs.	
<i>t</i>	Relative <i>n.</i>	<i>t</i>	Relative <i>n.</i>	<i>t</i>	Relative <i>n.</i>	<i>t</i>	Relative <i>n.</i>
60	2,280	60	2,297	60	2,707	60	2,700
90	2,288	90	2,298	90	2,710	90	2,701
150	2,304	150	2,285	150	2,693	120	2,703
Mean.....	2,291	Mean.....	2,293	Mean.....	2,703	Mean.....	2,701
Abnormal invertase M 2 + 10 cc. 0.01 per cent egg albumen solution.				Abnormal invertase M 1 + 10 cc. 0.01 per cent egg albumen solution.			
Experiment D 2. pH = 4.9.				Experiment D 5. pH = 4.9.			
I.P. = 0		I.P. = 4 hrs.		I.P. = 0		I.P. = 4 hrs.	
<i>t</i>	Relative <i>n.</i>	<i>t</i>	Relative <i>n.</i>	<i>t</i>	Relative <i>n.</i>	<i>t</i>	Relative <i>n.</i>
60	2,293	60	2,297	60	2,680	60	2,688
90	2,290	90	2,308	90	2,685	90	2,689
150	2,297	150	2,290	150	2,682	120	2,700
Mean.....	2,293	Mean.....	2,298	Mean.....	2,682	Mean.....	2,688
Abnormal invertase M 3.							
Experiment E 1. pH = 4.8.				Experiment E 5. pH = 4.8.			
I.P. = 0		I.P. = 4 hrs.		I.P. = 0		I.P. = 4 hrs.	
<i>t</i>	Relative <i>n.</i>	<i>t</i>	Relative <i>n.</i>	<i>t</i>	Relative <i>n.</i>	<i>t</i>	Relative <i>n.</i>
60	2,130	60	1,962	60	2,127	60	1,948
120	2,071	120	1,909	120	2,075	120	1,913
150	2,057	150		150	2,057		
First <i>n.</i> ...	2,130	First <i>n.</i> ...	1,962	First <i>n.</i> ...	2,127	First <i>n.</i> ...	1,948

TABLE II—*Concluded.*

Abnormal invertase M 3 + 0.1 cc. inactive normal invertase M.							
Experiment E 2. pH = 4.8.				Experiment E 6. pH = 4.8.			
I.P. = 0		I.P. = 4 hrs.		I.P. = 0		I.P. = 4 hrs.	
<i>t</i>	Relative n.	<i>t</i>	Relative n.	<i>t</i>	Relative n.	<i>t</i>	Relative n.
60	2,135	60	2,025	60	2,135	60	2,017
120	2,093	120	2,006	120	2,092	120	1,997
150	2,067			150	2,071		
First n....	2,135	First n...	2,025	First n...	2,135	First n...	2,017
Abnormal invertase M 3 + 1.0 cc. inactive normal invertase M.							
Experiment E 3. pH = 4.8.				Experiment E 7. pH = 4.8.			
I.P. = 0		I.P. = 4 hrs.		I.P. = 0		I.P. = 4 hrs.	
<i>t</i>	Relative n.	<i>t</i>	Relative n.	<i>t</i>	Relative n.	<i>t</i>	Relative n.
60	2,143	60	2,082	60	2,138	60	2,067
120	2,115	120	2,051	120	2,113	120	2,041
150	2,089			150	2,084		
First n....	2,143	First n..	2,082	First n..	2,138	First n..	2,067
Abnormal invertase M 3 + 5.0 cc. inactive normal invertase M							
Experiment E 4. pH = 4.9.				Experiment E 8. pH = 4.9.			
I.P. = 0		I.P. = 4 hrs.		I.P. = 0		I.P. = 4 hrs.	
<i>t</i>	Relative n.	<i>t</i>	Relative n.	<i>t</i>	Relative n.	<i>t</i>	Relative n.
60	2,150	60	2,102	60	2,153	60	2,112
120	2,141	120	2,093	120	2,141	120	2,108
150	2,121			150	2,133		
First n....	2,150	First n...	2,102	First n...	2,153	First n...	2,112

The method employed by the latter investigators in examining the stability of various invertase preparations, consisted essentially in allowing a solution of the enzyme preparation, containing no sucrose, to stand in a thermostat at 25°C. and a given pH (4.9) for 5 hours. This length of time corresponded to the time required

for the hydrolysis of a 10 per cent sucrose solution by means of the enzyme. The activity of the enzyme solution before and after standing for 5 hours (inactivation period) in the thermostat, was ascertained by adding a definite number of cubic centimeters of the enzyme solution to a sugar solution of such concentration that the resulting solution would contain 10 per cent sucrose, and from the rate of hydrolysis, calculate the relative values of the hydrolysis velocity constant, n , by means of the Nelson and Hitchcock equation. The loss in the magnitude of the constant, n , due to the 5 hour inactivation, was taken as a measure of the decrease in activity. The justification for considering the decrease in the value of n as a measure of the loss in activity was based on the observation made by Nelson and Hitchcock, that the value of n for a hydrolysis is proportional to the amount of invertase present in the hydrolyzing sugar solution. The procedure used throughout this paper in the study of relative stability of invertase solutions is essentially the same.

In order to see whether the abnormal invertase obtained by the aid of the iron sol from normal preparation could also be stabilized by the addition of normal invertase, inactivated by heating, the following experiments were undertaken in accordance with the procedure outlined above. A solution was made up of 12.5 cc. of abnormal invertase M, 2.5 cc. of 0.1 M citrate buffer, and 10 cc. of normal invertase M, which had been completely inactivated by heating. Another solution was made up of 12.5 cc. of abnormal invertase M, 2.5 cc. of 0.1 M citrate buffer, and 10 cc. of distilled water. The stability of both solutions was determined over a period of 4 hours. The results in Table II show that although abnormal invertase M lost 14.4 per cent of its value of n in 4 hours, the presence of 10 cc. of inactivated normal invertase M in 25 cc. of solution of abnormal invertase M resulted in complete restabilization of the abnormal preparation.

In order to see whether this restabilization was brought about by the addition of some substance, contained in the normal invertase preparation in higher concentration than in the abnormal preparation, the following experiment was tried. A solution was made up of 12.5 cc. of abnormal invertase M, 2.5 cc. of 0.1 M citrate buffer, and 10 cc. of abnormal invertase M, which had previously been completely inactivated by heating. The 10 cc.

portion of inactivated abnormal invertase used in this experiment possessed the same activity, that is the same value for n , previous to heating, as did the 10 cc. portion of inactivated normal invertase, previous to heating, that was used in the preceding experiment. The results given in Table II indicate that abnormal invertase is not restabilized by the addition of the inactivated abnormal invertase.

Effect of Gelatin and Egg Albumen on the Stability of Abnormal Invertase.

The accompanying material in invertase solutions is chiefly gums and proteins, colloidal in nature, and belongs to the class of substances generally known as protective colloids. This fact suggested that other protective colloids such as gelatin and egg albumen might also influence the stability of abnormal invertase. Consequently, to three 12.5 cc. portions of abnormal invertase, the following were added: To the first, 10 cc. of 0.01 per cent gelatin solution; to the second, 10 cc. of 0.01 per cent egg albumen; and to the last, 10 cc. of water. The three solutions each contained 2.5 cc. of 0.1 M citrate buffer of such composition as to give the desired pH.

The relative stability of the enzyme in each solution was determined in the manner described above. The results of these experiments, in Table II, show that the abnormal invertase was completely restabilized by gelatin and egg albumen.

Influence of the Concentration of a Normal Invertase Preparation, Which Had Been Inactivated by Heating, on the Degree of Stability of an Abnormal Preparation.

As has been shown by the results in Table II, 10 cc. of the inactivated normal preparation M added to 12.5 cc. of the abnormal preparation M, rendered the latter normal. In order to see whether still smaller amounts of the inactivated preparation would furnish this protective effect on the abnormal preparation, another series of experiments was undertaken. These experiments, in contrast to the above, involved the addition of 5.0, 1.0, 0.1, and 0.0 cc. of the inactivated normal preparation together with enough water to equal 10 cc. The rest of the procedure was the same.

The results obtained are recorded in Table II and show that there is a gradual decrease in the stabilizing effect as the amount of added inactivated material was decreased. As in the case of the experiments in the last two sections, the relative values of the sucrose hydrolysis velocity constants, n , were used as a measure of the protective influence of added inactivated normal preparation M. These decreases in n , corresponding to the above 5.0, 1.0, 0.1, and 0.0 cc. of added inactivated material, were respectively, 2.1, 3.1, 5.4, and 8.1 per cent during the inactivation period of 4 hours.

Effect of Dilution on the Stability of Invertase Preparation.

This protecting effect, therefore, appears to be a function of the concentration of the inactivated material in the solution containing the active abnormal preparation. Therefore, if a solution, containing a certain quantity of this protecting material associated with the active enzyme, were diluted, the concentration of the former would be decreased. This decrease in the concentration should, in the light of the above described experiments, tend to diminish the protecting effect, if the process of protection were reversible with dilution, and if the dilution were sufficiently great to render even a normal preparation abnormal. In order to obtain data on this point, the following experiments were run.

Immediately after experiments Nos. E 3 and E 7 were completed, the partially stabilized solution, containing 1.0 cc. of added inactivated normal invertase preparation M per 25 cc., was diluted ten times with water and citrate buffer, and the stability of this solution measured in the usual manner. The results of this experiment, given in Table III, show that this diluted sample now lost 10.6 per cent in the value of n in 4 hours, whereas previous to dilution it had lost only 3.1 per cent in the value of n during the same period of time. These results indicate that the process of stabilization is reversible with dilution.

Effect of Dilution on the Stability of Normal Invertase.

A sample of invertase M was diluted 200 times with water and citrate buffer to give the solution a pH of about 4.5. The stability of the invertase in this solution, together with that of another portion of normal invertase M, undiluted except for enough citrate

TABLE III.

Reversibility of the Stabilization of Abnormal Invertase with Dilution.

Experiment F 1. Reaction of solution of Experiment E 3 diluted 10 times. pH = 4.7.				Experiment F 2. Reaction solution of Experiment E 7 diluted 10 times. pH = 4.7.			
I.P. = 0		I.P. = 4 hrs.		I.P. = 0		I.P. = 4 hrs.	
<i>t</i>	Relative <i>n.</i>	<i>t</i>	Relative <i>n.</i>	<i>t</i>	Relative <i>n.</i>	<i>t</i>	Relative <i>n.</i>
240	1,926	240	1,711	240	1,954	240	1,758
300	1,852	300	1,648	300	1,882	300	1,695
First <i>n</i> ...	1,926	First <i>n</i> ..	1,711 Loss in <i>n</i> = 11.2 per cent.	First <i>n</i> .	1,954	First <i>n</i> ..	1,758 Loss in <i>n</i> = 10.0 per cent.

TABLE IV.

Restabilization of Normal Invertase Preparation Rendered Unstable by Dilution.

Normal invertase M diluted 200 times.							
Experiment G 1. pH = 4.7.				Experiment G 5. pH = 4.7.			
I.P. = 0		I.P. = 48 hrs.		I.P. = 0		I.P. = 48 hrs.	
<i>t</i>	Relative <i>n.</i>	<i>t</i>	Relative <i>n.</i>	<i>t</i>	Relative <i>n.</i>	<i>t</i>	Relative <i>n.</i>
1,000	2,865	1,000	2,625	1,000	2,877	1,000	2,561
1,200	2,684	1,200	2,585	1,200	2,771	1,200	2,470
1,440	2,656	1,440	2,527	1,440	2,672	1,440	2,379
First <i>n</i> ...	2,865	First <i>n</i> ..	2,625 Loss in <i>n</i> = 8.4 per cent.	First <i>n</i> .	2,877	First <i>n</i> ..	2,561 Loss in <i>n</i> = 11.0 per cent.

Normal invertase M diluted 200 times, containing 25 cc inactive normal invertase M per 100.							
Experiment G 2. pH = 4.8.				Experiment G 6. pH = 4.8.			
I.P. = 0		I.P. = 48 hrs.		I.P. = 0		I.P. = 48 hrs.	
<i>t</i>	Relative <i>n.</i>	<i>t</i>	Relative <i>n.</i>	<i>t</i>	Relative <i>n.</i>	<i>t</i>	Relative <i>n.</i>
1,000	3,308	1,000	3,431	1,000	3,290	1,000	3,363
1,200	3,285	1,200	3,420	1,200	3,257	1,200	3,358
1,440	3,206	1,440	3,398	1,440	3,202	1,440	3,298
First <i>n</i>	3,308	First <i>n</i> ..	3,431	First <i>n</i> ..	3,290	First <i>n</i> ..	3,363

TABLE IV—*Continued*

Normal invertase M diluted 200 times, containing 25 cc. 0.1 per cent gelatin solution per 100.

Experiment G 3. pH = 4.9.				Experiment G 7. pH = 4.9.			
I.P. = 0		I.P. = 48 hrs.		I.P. = 0		I.P. = 48 hrs.	
<i>t</i>	Relative <i>n.</i>	<i>t</i>	Relative <i>n.</i>	<i>t</i>	Relative <i>n.</i>	<i>t</i>	Relative <i>n.</i>
1,000	3,590	1,000	3,613	1,000	3,563	1,000	3,577
1,220	3,600	1,200	3,603	1,200	3,573	1,200	3,578
1,440	3,594	1,440	3,612	1,440	3,564	1,440	3,577
Mean . . .	3,595	Mean . . .	3,609	Mean . . .	3,567	Mean . . .	3,577

Normal invertase M diluted 200 times, containing 25 cc. 0.1 per cent egg albumen solution per 100.

Experiment G 4. pH = 4.8.				Experiment G 8. pH = 4.8.			
I.P. = 0		I.P. = 48 hrs.		I.P. = 0		I.P. = 48 hrs.	
<i>t</i>	Relative <i>n.</i>	<i>t</i>	Relative <i>n.</i>	<i>t</i>	Relative <i>n.</i>	<i>t</i>	Relative <i>n.</i>
1,000	3,594	1,000	3,601	1,000	3,569	1,000	3,565
1,220	3,600	1,200	3,607	1,200	3,583	1,200	3,571
1,440	3,601	1,440	3,600	1,440	3,580	1,440	3,577
Mean	3,598	Mean . . .	3,603	Mean . . .	3,577	Mean . . .	3,571

buffer to bring the pH to 4.5, were measured in the above described manner. The results given in Table IV show that the diluted normal invertase M lost 9.7 per cent of its value of *n* during an inactivation period of 48 hours. Undiluted invertase M showed no decrease in its value of *n* even after an inactivation period of 1 week.

It is evident that if the cause of the loss in activity on dilution of a normal preparation is essentially the same as the cause of the loss in activity manifested by an abnormal preparation, prepared from a normal by the removal of certain accompanying material, then the same conditions which produced stability in one case should produce stability in the other; namely, the addition of inactivated normal invertase preparation, gelatin, and egg albumen. Accordingly, three samples of normal invertase M were made up so that it would be diluted 200 times in the final reaction mixture. One contained 25 cc. of normal invertase M, previously

completely inactivated by heating; another, 25 cc. of 0.1 per cent gelatin solution; and the last, 25 cc. of 0.1 per cent egg albumen solution per 100 cc. of the solution subjected to inactivation. Citrate buffer was present in each solution in 0.01 M concentration, and of such composition as to give the desired pH. The results, given in Table IV, of the determination of the stability of each of the above solutions of invertase, are in agreement with this idea. Although normal invertase M showed a loss of 9.7 per cent in the value of n in 48 hours on diluting 200 times, the addition of either normal invertase preparation, inactivated by heating, gelatin, or egg albumen prevented this loss. Thus we may conclude, that instability on dilution and abnormality have for their explanation the same underlying principle; namely, an insufficiency of stabilizing material accompanying the enzyme.

That invertase solutions are subject to loss in activity on dilution was observed by Vosburgh (4). He reported that three invertase preparations, when diluted considerably, all became unstable, and that the magnitude of the activity lost varied for different invertase preparations under the same conditions of experiment. This is what would be expected on the basis of the above explanation, since in general, no two invertase solutions contain the same concentration of solid invertase preparation, nor do the latter possess identical composition.

The fact that invertase requires a certain concentration of stabilizing material to render it normal at pH 4.5 and 25°C. suggests why Nelson and Hitchcock had difficulty in stabilizing their abnormal preparation No. 6 by the same manner in which abnormal preparation No. 3 was stabilized; namely, the addition of 10 cc. of inactivated normal invertase preparation No. 8 per 100 cc. of reaction mixture. In looking over the data given in their Tables VIII and X, which are summarized in Table V, No. 3 appears to have lost on standing in the ice box only 2 per cent of its activity in 5 months, while No. 6 during the same period lost 6.5 per cent, when the sucrose hydrolysis velocity constant, n , at $t=15$ minutes is taken as a measure of their relative activities. That the greater stability of No. 3 was due to the presence of a greater concentration of stabilizing material is indicated by the following. During the course of the sucrose hydrolysis, No. 3 lost 4.9 per cent in the value of n from $t=15$ minutes to $t=300$,

while No. 6 lost 6.9 per cent during the same time interval. Furthermore, No. 6 was so active that only 1.905 cc. of the prepara-

TABLE V.
Data of Nelson and Hitchcock.

Experiments B 12 to 15 * 1 905 cc. invertase 3 per 100			Experiments B 17 and 18.* 10 45 cc. invertase 6 per 100.		
<i>t</i>	<i>p</i> , per cent inverted	<i>n</i> $\times 10^3$	<i>t</i>	<i>p</i> , per cent inverted.	<i>n</i> $\times 10^3$
5	3 20	450	15	10 03	476
10	6 35	449	30	19.41	470
15	9 44	448	50	31 16	466
22	13 65	445	70	41 84	461
30	18 46	446	90	51 34	457
60	34 96	440	115	61 72	454
90	49 38	436	140	70 15	451
120	61 48	433	180	80 30	448
180	78 81	431	240	89 67	449
300	93 59	426	300	94 30	443
Mean... 440 \pm 1.4%			Mean... 457 \pm 1.9%		
Decrease in <i>n</i> (from <i>t</i> = 15 to 300) = 4.9 per cent.			Decrease in <i>n</i> (from <i>t</i> = 15 to 300) = 6.9 per cent.		
Experiment B 25 † 1 905 cc invertase 3 per 100 + 10 cc inactive invertase 8 per 100			Experiments B 35 and B 37 † 10 45 cc invertase 6 per 100 + 10 cc inactive invertase 8 per 100.		
<i>t</i>	<i>p</i> , per cent inverted	<i>n</i> $\times 10^3$	<i>t</i>	<i>p</i> , per cent inverted	<i>n</i> $\times 10^3$
15	9 26	439	15	9.38	445
30	18 22	440	30	18.34	443
60	34 78	437	60	34.84	438
90	49 38	436	90	49.20	434
120	61 66	434	120	61 25	430
180	79 29	437	180	78.52	428
240	89 14	440	240	88.43	429
300	94 07	437	300	93.53	425
Mean... 437 \pm 0.34%			Mean... 434 \pm 1.38%		

* Taken from Nelson and Hitchcock's Table VIII.

† Taken from Nelson and Hitchcock's Table X.

The experiments in Table X were run about 5 months after the experiments in Table VIII, the invertase preparations remaining in the ice box during this interval.

tion were used per 100 cc. of hydrolysis solution, whereas in the case of No. 3, 10.45 cc. were required. In other words, No. 3 had been diluted over 50 times with sugar solution, whereas No. 6 had been diluted less than ten times to produce even a greater loss in the value of n . Nelson and Hitchcock also state that preparation No. 3 could be made normal by concentrating it about three times by evaporation, while concentrating No. 6 this amount did not normalize it. Lastly, the presence of 10 cc. of normal invertase No. 8, inactivated by heating, in 100 cc. of sugar solution, in which were also present 1.905 cc. of No. 6, did eliminate some of the abnormality of the latter and decreased its average deviation from the mean n from 1.9 to 1.38 per cent. The presence of 10 cc. of inactivated normal invertase preparation No. 8 in 100 cc. of sugar solution, in which were also present 10.45 cc. of No. 3, rendered No. 3 normal. This behavior of Nos. 3 and 6 points to the probability that No. 3 was nearer normal to begin with than No. 6.

Comparative Stabilities of Normal and Abnormal Invertase Preparations at Higher Acidities.

Bloomfield (5) observed that at 25°C. normal invertase preparations showed a tendency to give decreasing sucrose hydrolysis constants, n , as the acidity of the solution containing sucrose was increased beyond a value of pH 2.75. Nelson and Hollander found that normal invertase preparations were unstable at pH 2.4 and 25°C. but that the abnormal preparations Nos. 3 and 6 of Nelson and Hitchcock were far more unstable under the same conditions. This difference in stability in the acid region has been found to be so also for normal invertase M, when compared with that of the abnormal preparation M. The latter was prepared, as mentioned above, from the normal preparation by means of treatment with iron sol. The procedure followed in ascertaining this last point was the same as that adopted in the study of stability at pH 4.5, the only difference being that the enzyme solutions were subjected to longer inactivation periods; that is, 25 hours instead of 4. The results of these experiments are given in Tables VI and VII.

It was found that the presence of varying amounts of normal preparation M, previously inactivated by heating, in the solu-

TABLE VI.

Experiment No.	Concentration of inactive invertase preparation added	Inactivation period	Relative hydrolysis velocity constant, n .	Loss, n .
		<i>hrs.</i>		<i>per cent</i>
H 1	0 0	0 25	815 375	54 0
H 2	4 0 cc. normal.	0 25	804 388	51 8
H 3	6 0 " "	0 25	815 414	49 2
H 4	8 0 " "	0 25	818 437	46 6
H 5	10 0 " "	0 25	826 474	42 6
H 6	12 0 " "	0 25	825 485	40 1
H 7	20 0 " "	0 25	818 544	33 5
H 8	40 0 " "	0 25	828 617	25 5
H 9	20 0 " abnormal.	0 25	860 380	56 2

Experiment H 1 Abnormal invertase M 6 pH = 2.2				Experiment H 2 Abnormal invertase M 6 + 4 cc inactive normal invertase M. pH = 2.2			
I P. = 0		I P. = 25 hrs		I P. = 0		I P. = 25 hrs.	
t	Relative n	t	Relative n	t	Relative n	t	Relative n .
60	815	90	375	60	804	90	388
120	792	170	370	120	790	170	380
180	777			180	781		
First n ...	815	First n .	375	First n .	804	First n ..	388

TABLE VI—*Concluded.*

Experiment H 3. Abnormal invertase M 6 + 6 cc. inactive normal invertase M. pH = 2.2.				Experiment H 4. Abnormal invertase M 6 + 8 cc. inactive normal invertase M. pH = 2.2.			
I.P. = 0		I.P. = 25 hrs.		I.P. = 0		I.P. = 25 hrs.	
<i>t</i>	Relative <i>n.</i>	<i>t</i>	Relative <i>n.</i>	<i>t</i>	Relative <i>n.</i>	<i>t</i>	Relative <i>n.</i>
60	815	90	414	60	818	90	436
120	809	170	411	120	810	170	438
180	802			180	804		
First <i>n.</i> ...	815	First <i>n.</i> ..	414	First <i>n.</i>	818	First <i>n.</i> ..	436
Experiment H 5. Abnormal invertase M 6 + 10 cc. inactive normal invertase M. pH = 2.2.				Experiment H 6. Abnormal invertase M 6 + 12 cc. inactive normal invertase. pH = 2.2.			
I.P. = 0		I.P. = 25 hrs.		I.P. = 0		I.P. = 25 hrs.	
<i>t</i>	Relative <i>n.</i>	<i>t</i>	Relative <i>n.</i>	<i>t</i>	Relative <i>n.</i>	<i>t</i>	Relative <i>n.</i>
60	826	90	471	60	826	90	485
120	823	170	477	120	824	170	485
180	821			180	824		
First <i>n.</i> ...	826	Mean...	474	Mean <i>n.</i> ..	825	Mean...	485
Experiment H 7. Abnormal invertase M 6 + 20 cc. inactive normal invertase M. pH = 2.2.				Experiment H 8. Abnormal invertase M 6 + 40 cc. inactive normal invertase. pH = 2.2			
I.P. = 0		I.P. = 25 hrs.		I.P. = 0		I.P. = 25 hrs.	
<i>t</i>	Relative <i>n.</i>	<i>t</i>	Relative <i>n.</i>	<i>t</i>	Relative <i>n.</i>	<i>t</i>	Relative <i>n.</i>
60	818	90	543	60	829	90	617
120	821	170	545	120	827	170	617
180	816			180	829		
Mean <i>n.</i> ...	818	Mean ...	544	Mean...	828	Mean....	617
Experiment H 9. Abnormal invertase M 6 + 20 cc. inactive abnormal invertase M.							
I.P. = 0		I.P. = 25 hrs.					
<i>t</i>	Relative <i>n.</i>	<i>t</i>	Relative <i>n.</i>				
60	869	90	380				
120	849	170	376				
180	840						
First <i>n.</i> ...	869	First <i>n.</i> ..	380				

tions of the abnormal preparation M, while the latter were being subjected to inactivation by more acid solutions, showed again a gradual protecting influence. This protecting influence of inacti-

TABLE VII

Experiment No	Concentration in cc of added inactive invertase preparation per 100	Inactivation period	Relative hydrolysis velocity constant, n	Loss in n
				<i>per cent</i>
I 1	0 0	0	1,889	
		25	1,621	14 2
I 2	5 cc normal	0	1,890	
		25	1,531	19 0
I 3	10 " "	0	1,891	
		25	1,497	20 8
I 4	15 " "	0	1,930	
		25	1,507	21 9
I 5	20 " "	0	1,918	
		25	1,487	22 5
I 6	25 " "	0	1,950	
		25	1,499	23 1
I 7	35 " "	0	1,955	
		25	1,496	23 5
I 8	30 " abnormal	0	1,899	
		25	1,622	14 6

Normal invertase M Experiment I 1 pH = 2.2				Normal invertase M + 5 cc inactive undiluted normal invertase M Experiment I 2 pH = 2.2			
I P = 0		I P = 25 hrs		I P = 0		I P = 25 hrs	
t	Relative n	t	Relative n	t	Relative n	t	Relative n
90	1,890	90	1,621	90	1,889	90	1,527
105	1,883	105	1,620	105	1,892	105	1,532
120	1,895	120	1,621	120	1,889	120	1,535
Mean.	1,889	Mean	1,621	Mean	1,890	Mean	1,531

TABLE VII—*Concluded.*

Normal invertase M + 10 cc. inactive undiluted normal invertase M. Experiment I 3. pH = 2.2.				Normal invertase M + 15 cc. inactive undiluted normal invertase M. Experiment I 4. pH = 2.2.			
I.P. = 0		I.P. = 25 hrs.		I.P. = 0		I.P. = 25 hrs.	
<i>t</i>	Relative <i>n.</i>	<i>t</i>	Relative <i>n.</i>	<i>t</i>	Relative <i>n.</i>	<i>t</i>	Relative <i>n.</i>
90	1,898	90	1,496	90	1,930	90	1,502
105	1,891	105	1,497	105	1,929	105	1,509
120	1,883	120	1,497	120	1,930	120	1,509
Mean.....	1,891	Mean....	1,497	Mean....	1,930	Mean....	1,507
Normal invertase M + 20 cc. inactive undiluted normal invertase M. Experiment I 5. pH = 2.2.				Normal invertase M + 25 cc. inactive undiluted normal invertase M. Experiment I 6. pH = 2.2.			
I.P. = 0		I.P. = 25 hrs.		I.P. = 0		I.P. = 25 hrs.	
<i>t</i>	Relative <i>n.</i>	<i>t</i>	Relative <i>n.</i>	<i>t</i>	Relative <i>n.</i>	<i>t</i>	Relative <i>n.</i>
90	1,916	90	1,479	90	1,942	90	1,480
105	1,922	105	1,492	105	1,953	105	1,512
120	1,916	120	1,491	120	1,955	120	1,504
Mean.....	1,918	Mean....	1,487	Mean....	1,950	Mean....	1,499
Normal invertase M + 35 cc. inactive undiluted normal invertase M. Experiment I 7. pH = 2.2.				Normal invertase M + 30 cc. inactive undiluted abnormal invertase M. Experiment I 8. pH = 2.2.			
I.P. = 0		I.P. = 25 hrs.		I.P. = 0		I.P. = 25 hrs.	
<i>t</i>	Relative <i>n.</i>	<i>t</i>	Relative <i>n.</i>	<i>t</i>	Relative <i>n.</i>	<i>t</i>	Relative <i>n.</i>
90	1,960	90	1,485	90	1,900	90	1,624
105	1,953	105	1,497	105	1,896	105	1,622
120	1,952	120	1,506	120	1,901	120	1,621
Mean.....	1,955	Mean....	1,496	Mean....	1,899	Mean....	1,622

vated normal on the stability of abnormal preparation at pH 2.2 is, therefore, like that observed at pH 4.5, already described above. The following series of solutions containing active abnormal invertase M and varying amounts of normal invertase M, previously inactivated by heating, was made up. These solutions contained 0, 4.0, 6.0, 8.0, 10.0, 12.0, 20.0, and 40.0 cc. of

inactivated normal invertase per 100 cc. of reaction solution. They showed a progressive decrease in the loss in the value of n as follows: 54.0, 51.8, 49.2, 46.6, 42.6, 40.1, 33.5, and 25.5 per cent, respectively, in 25 hours at pH 2.2 and 25°C. Furthermore, in contrast to the effect of the inactivated normal invertase, the addition of abnormal invertase preparation M, previously inactivated by heating, failed to show, just as it did at pH 4.5, this protecting influence on abnormal invertase preparation. For the results of these experiments see Table VI.

The results in Table VII show that the inactivation of the normal preparation M by acid at pH 2.2 could not be decreased by the presence of more normal preparation, which had been inactivated by heating. These results also reveal the interesting fact that there is a marked increase in the rate of inactivation of normal invertase M, depending on the concentration of added inactive material. In 25 hours, invertase M, without the addition of inactivated material, lost only 14.2 per cent of its activity, whereas the addition of 5, 10, 15, 20, 25, and 35 cc. of inactivated, undiluted normal invertase M in separate 100 cc. portions of the reaction solution, caused the percentage loss in the value of n to increase to 19.0, 20.8, 21.9, 22.5, 23.1, and 23.5 per cent, respectively. This effect was not observed at pH 4.5.

It was observed further, that just as inactivated abnormal invertase had no protecting effect on active abnormal invertase, inactivated abnormal invertase had no inactivating effect on normal invertase. Thus a solution of normal invertase M, containing 30 cc. of inactivated abnormal invertase M per 100 cc. of reaction mixture, lost only 14.6 per cent of its value of n under the same conditions as above. This value is practically the same as that obtained above for normal invertase M by itself; namely, 14.2 per cent. This experiment seems to indicate that this increase in the rate of inactivation of normal invertase M, mentioned in the last paragraph, cannot be due to the presence of the inactivated invertase itself, but rather to the presence of some other substance present in normal preparation, but absent in the abnormal.

On recapitulation, the above experimental results show that the instability of the invertase preparation studied, in the acid region, pH 2.2, is subject to, at least, three conditions. First, the

TABLE VIII.

Effect of Dilution on the Rate of Inactivation of Invertase at pH 2.2 and 25°C.

Inactivation period.	Relative sucrose hydrolysis velocity constant, n .		Average relative n .	Loss in n .	$k \times 10^4$ *
	Experiment J 1.	Experiment J 2.			
Inactivation using 25 cc. of Preparation M per 100 cc. of reaction solution					
				<i>per cent</i>	
0	1,962	1,962	1,962		
3.5	1,723	1,708	1,716	12.5	165.7
25	1,485	1,520	1,503	23.4	46.3
48	1,470	1,457	1,464	24.9	25.9
72	1,377	1,329	1,353	31.0	22.4
96	1,290	1,221	1,256	36.0	20.2
144	1,170	1,143	1,157	41.0	15.9
240	970	972	971	50.5	12.7
432	742	751	747	61.9	9.4

Inactivation using 12.5 cc. of Preparation M per 100 cc. of reaction solution.

	Experiment J 3.	Experiment J 4.			
0	951	951	951		
25	810	809	810	14.9	
48	755	757	756	20.5	
72	699	687	693	27.1	
96	631	625	628	34.0	
144	580	589	587	38.3	
240	455	459	457	52.0	
432	323	324	324	66.0	

Inactivation using 2.5 cc. of Preparation M per 100 cc. of reaction solution.

	Experiment J 5.	Experiment J 6.	Experiment J 7.			
0	188.9	191.5	190.4	190.3		
3.5	181.6	185.8	183.3	183.6	3.5	44.3
25	162.1	162.9	161.5	162.2	14.7	27.6
48	147.3	146.5	145.6	146.5	23.0	23.6
72	132.1	132.9	130.7	131.9	30.7	22.1
96	122.4	121.2	120.4	121.3	36.3	20.4
144	108.1	107.3	106.7	107.4	43.0	17.0
240	82.2	85.3	81.9	83.1	56.3	15.0
432	57.7	55.2	55.7	56.2	70.5	12.3

* k , the monomolecular reaction constant for the inactivations, was calculated by means of the equation, $k = \frac{1}{t} \log \frac{100}{100-p}$, where p is the percentage loss in n at time t .

instability of abnormal preparations can be decreased by the presence of stabilizing material. Second, the instability of normal preparations cannot be decreased. Third, the instability of normal preparations is increased by the presence of an excess of normal invertase preparation, previously inactivated by heating. The second and third statements are difficult to reconcile, unless it is assumed that something exists in normal invertase preparations, which when present in sufficient amount tends to inactivate invertase under these conditions of pH and temperature.

Nelson and Hollander (2) showed, although their results were not described with this idea in mind, that the stability of different normal invertase preparations, namely Nos. 7 and 8, was different in the acid region under the same conditions of pH and temperature. During an inactivation period of 5 hours, No. 7 lost 14.5 per cent in its value of n , while No. 8 lost only 4.2 per cent. It is evident in the light of the experiments, described in the preceding paragraphs, that this might be expected, because it is well known that the various accompanying materials differ in the amounts present in the different invertase preparations.

Hudson and Paine (6) reported that the inactivation of invertase was a reaction of the first order. Euler (7), however, obtained decreasing monomolecular reaction constants in every case, and concludes that the simple course of the reaction obtained by previous workers obtains only under special conditions. If, as Hudson and Paine claim, the inactivation of invertase by acid follows the monomolecular reaction law, then the dilution of invertase, keeping the pH constant, should not change the inactivation curve, when the percentage inactivation is plotted against time. But if the stability of invertase is, in a measure, determined by the concentration of accompanying material in the solution, then inactivating invertase, at various invertase preparation concentrations, should change the shape of the inactivation curve. To show that this was actually so, normal invertase M was inactivated at three different dilutions. One contained 25 cc. of undiluted invertase preparation M per 100 cc. of the inactivation solution; the second, 12.5 cc.; and the third, 2.5 cc. The pH was the same for all three solutions. The results in Table VIII, where the percentage loss in the sucrose hydrolysis velocity constant, n , is given for

various time intervals in the course of the inactivation, show that the course of the inactivation is not comparable for any two dilutions. It will be seen that over the first part of the inactivation, the more concentrated invertase preparation solutions inactivate more readily, whereas in the latter part of the inactivation, the more dilute inactivate faster.

It will be seen that on applying the monomolecular reaction equation to the results of the inactivations of invertase M, given in Table VIII, where the activity of the solutions was calculated at various times during the course of the reaction, on the basis of n , the sucrose hydrolysis velocity constant, the monomolecular constants of the inactivation decrease very markedly. These results substantiate Euler's claim.

It was observed that when invertase solutions, concentrated with respect to the invertase preparation content, were inactivated in the acid region and then brought back to pH 4.5 to measure the activity in the manner above described, there was an increase in n during the course of the sucrose hydrolysis, indicating that there might be a slight reversibility in the inactivation of such solutions. Since this effect was not observable in the case of more dilute solutions it was assumed that this effect was due to a reversibility of that part of the inactivation due to an excess of material accompanying the enzyme, or more specifically, that material which may cause an increase in the instability of invertase. Advancing on this assumption, two solutions of invertase were inactivated at pH 2.2 for 5 hours, one containing 40 cc. of undiluted normal invertase M per 100 cc. of reaction solution, the other 8 cc. At the end of 5 hours, the former was diluted ten times, the latter twice, with water and citrate buffer to bring the pH to about 4.5. The activity of each of these solutions was determined immediately and again at the end of 2 hours. The results, given in Table IX, show that the more concentrated solution lost 14.2 per cent of its value of n in 5 hours at pH 2.2, while the less concentrated lost only 5.4 per cent. On dilution and bringing the pH back to 4.5, however, the more concentrated solution, on standing for 2 hours before adding it to the sucrose solution, had gained 3.1 per cent in its value of n , whereas the less concentrated one suffered practically no change in the value of n .

TABLE IX.
Reversible Inactivation: Abnormality of a Second Type.

Experiment K 1 Normal invertase M, 40 cc per 100 pH = 2.2 pH of diluted solution = 4.7					
IP = 0		IP = 5 hrs		IP = 7 hrs	
<i>t</i>	Relative <i>n</i>	<i>t</i>	Relative <i>n</i>	<i>t</i>	Relative <i>n</i>
30	3,053	30	2,618	30	2,717
45	3,057	45	2,631	45	2,693
60	3,060	60	2,632	60	2,697
Mean	3,057	First <i>n</i> 2,618 Loss in <i>n</i> = 14.3 per cent		First <i>n</i> 2,717 Loss in <i>n</i> = 11.1 per cent	

Experiment K 2 Normal invertase M, 8 cc per 100 pH = 2.2. pH of diluted solution = 4.6					
IP = 0		IP = 5 hrs		IP = 7 hrs	
<i>t</i>	Relative <i>n</i>	<i>t</i>	Relative <i>n</i>	<i>t</i>	Relative <i>n</i>
30	3,067	30	2,898	30	2,912
45	3,064	45	2,900	45	2,900
60	3,060	60	2,895	60	2,900
Mean	3,064	Mean 2,898 Loss in <i>n</i> = 5.4 per cent		Mean 2,904 Loss in <i>n</i> = 5.2 per cent.	

Experiment K 3 Normal invertase M, 40 cc per 100 pH = 2.2 pH of diluted solution = 4.6					
IP = 0		IP = 5 hrs		IP = 7 hrs	
<i>t</i>	Relative <i>n</i>	<i>t</i>	Relative <i>n</i>	<i>t</i>	Relative <i>n</i>
30	3,053	30	2,625	30	2,688
45	3,057	45	2,631	45	2,667
60	3,057	60	2,630	60	2,670
Mean	3,056	First <i>n</i> 2,625 Loss in <i>n</i> = 14.1 per cent		First <i>n</i> 2,688 Loss in <i>n</i> = 12.0 per cent.	

The hydrolyses in this table were all run at the same invertase preparation concentration.

Comparative Stabilities of Normal and Abnormal Invertase Preparations at Higher Temperatures.

Hudson and Paine (8) have contended that the same facts which explain the instability of invertase solutions to acid at lower temperatures, explain the instability of invertase solutions at higher temperatures. That is, the inactivation reaction is the same in both cases. The results, Table X, obtained by subjecting invertase preparation M to 60 and 65°, show that at these temperatures the abnormal preparation is fully as stable as the normal. This similarity in stability of normal and abnormal preparations is quite different from that at 25°, since at the latter temperature only the abnormal preparation was inactivated at pH 4.5, and at pH 2.2 the abnormal was inactivated to a greater extent than the normal.

The procedure followed was essentially the same as that adopted in the study of stability of invertase at 25°, which was described in the first section, with the following modifications. At 60° the inactivation period was limited to 1 hour and at 65° to 30 minutes. Immediately after the solutions of invertase had been made up, a portion from each was removed and added to a sucrose solution in order to determine its degree of activity before being subjected to higher temperatures. The remaining portions were immediately placed in a thermostat at the desired temperature. At the end of the inactivation period the solutions were removed and chilled to 25° in 3 minutes and the activities were again determined in the usual manner.

EXPERIMENTAL DETAILS.

Preparation of Materials.

Preparation of Ferric Oxide Hydrosol.—A solution of 0.1 M ammonium hydroxide added, drop by drop, with vigorous stirring to 100 cc. of M ferric chloride, until the precipitate formed, failed to redissolve with ease. The solution was then filtered and dialyzed in collodion bags for 48 hours against running tap water and finally for 12 hours against distilled water, which was changed every half hour. This solution was filtered and kept, undiluted in glass-stoppered bottles, till needed.

Preparation of Invertase Solutions.—Invertase K was prepared as follows: The autolyzed yeast juice from brewer's bottom yeast was precipitated with 1.5 times its volume of 95 per cent alcohol.

TABLE X.

Comparative Stabilities of Normal and Abnormal Invertase at 60 and 65°C.

Experiment No.	Solution.	Loss in η at 60° in 1 hr.	Loss in η at 65° in 0.5 hr.
	"	per cent	per cent
L 1	Normal invertase M.	48.1	
L 2	" " "	47.4	
L 3-4	Abnormal invertase M.	46.4	
L 5-6	" " "	46.9	
L 7	" " "		97.6
L 8	" " "		98.1
L 9	Normal invertase M.		100
L 10	" " "		100

Normal invertase M. Temperature = 60°C.

Experiment L 1. pH = 4.8.				Experiment L 2. pH = 4.8.			
I.P. = 0		I.P. = 1 hr.		I.P. = 0		I.P. = 1 hr	
t	Relative η .	t	Relative η .	t	Relative η .	t	Relative η .
90	2,976	90	1,531	90	2,616	90	1,388
120	2,976	120	1,523	120	2,618	120	1,370
150	2,977	150	1,528	150	2,618	150	1,373
Mean	2,976	Mean . . .	1,527	Mean	2,617	Mean	1,377

Temperature = 60°C.

Abnormal invertase M 2.				Abnormal invertase M 4.			
Experiment L 3. pH = 4.8.				Experiment L 5. pH = 4.8.			
I.P. = 0		I.P. = 1 hr.		I.P. = 0		I.P. = 1 hr.	
t	Relative η .	t	Relative η .	t	Relative η .	t	Relative η .
90	2,437	90	1,307	90	2,111	90	1,120
120	2,417	120	1,291	120	2,104	120	1,113
150	2,403			150	2,090		
First η . . .	2,437	First η . .	1,307	First η . .	2,111	First η . .	1,120

TABLE X—*Concluded.*

Temperature = 25°C.

Abnormal invertase M 2.				Abnormal invertase M 4.			
Experiment L 4. pH = 4.8.				Experiment L 6. pH = 4.8.			
I.P. = 0		I.P. = 1 hr.		I.P. = 0		I.P. = 1 hr.	
<i>t</i>	Relative <i>n.</i>	<i>t</i>	Relative <i>n.</i>	<i>t</i>	Relative <i>n.</i>	<i>t</i>	Relative <i>n.</i>
90	2,405	90	2,340	90	2,118	90	2,056
120	2,387	120	2,319	120	2,095	120	2,034
First <i>n.</i> ...	2,405	First <i>n.</i> ...	2,340	First <i>n.</i> ...	2,118	First <i>n.</i> ...	2,056

Temperature = 65°C.

Abnormal invertase M 5. pH = 4.8. Experiment L 7.					Abnormal invertase M 5. pH = 4.8. Experiment L 8.				
I.P. = 0		I.P. = 0.5 hr.			I.P. = 0		I.P. = 0.5 hr.		
<i>t</i>	Relative <i>n.</i>	<i>t</i>	<i>R</i>	Relative <i>n.</i>	<i>t</i>	Relative <i>n.</i>	<i>t</i>	<i>R</i>	Relative <i>n.</i>
		0	25.89				0	25.90	
60	1,695	60	25.83		60	1,670	60	25.83	
150	1,675	240	25.45		150	1,626	240	25.47	
210	1,654	1,320	23.38	40.9	210	1,601	1,320	23.95	31.2
	1,695			40.9		1,670			31.2

Temperature = 65°C.

Normal invertase M. Experiment L 9. pH = 4.8.					Normal invertase M. Experiment L 10. pH = 4.8.				
<i>t</i>	Relative <i>n.</i>	<i>t</i>	<i>R</i>	Relative <i>n.</i>	<i>t</i>	Relative <i>n.</i>	<i>t</i>	<i>R</i>	Relative <i>n.</i>
		0	25.90				0	25.90	
60	1,735	60	25.91	0.0	60	1,757	60	25.90	0.0
151	1,746	240	25.89	0.0	150	1,750	240	25.89	0.0
210	1,721				210	1,755			
	1,734			0.0		1,754			0.0

The gum was taken up with a volume of distilled water equal to the original volume of yeast juice. The process was then repeated, taking up the gum the second time, however, in half the quantity

of water used the first time. This solution was now dialyzed in collodion bags of 200 cc. capacity, against running tap water for 5 days and against distilled water for 24 hours. Invertase M was prepared by D. P. Morgan of this laboratory by the same method with the exception that the second precipitation with alcohol was omitted. Invertase 8 D, a separate portion of invertase No. 8, which had been placed in a smaller bottle of convenient size, was prepared by Nelson and Hitchcock (1), according to the method of Nelson and Born (3), with the following modifications. Only one precipitation with alcohol was used and the kaolin treatment was omitted. After treatment with lead acetate and potassium

TABLE XI.

Concentration of Sol and Invertase Preparation Used in the Treatment of Invertase by Ferric Oxide Hydrosol

Preparation No	Invertase preparation per 100	Citrate buffer per 100	Sol per 100
	<i>cc</i>	<i>cc</i>	<i>cc</i>
8 D	50	10	25
K	75	10	15
M	25	10	10

Increase in the Activity per Unit of Dry Weight of Invertase Preparation on Treatment with Ferric Oxide Hydrosol

Preparation No.	Sucrose hydrolysis velocity constant, \bar{n} , determined in using 0.01 gm. of solid preparation per 100 cc. of hydrolysis mixture	
	Before treatment with sol	After treatment with sol
M	0.00583	0.01122 0.01529
K	0.00221	0.00721 0.00540

oxalate, the filtrate was dialyzed for from 4 to 6 days in collodion bags against running tap water. The dialyzed solutions were not precipitated again. A fresh lot of brewer's bottom yeast was used in the preparation of both No. 8 and M.

Procedures.

Procedure for Preparing Abnormal Invertase.—Three series of invertase solutions were made up as follows: The first series was made up by taking 25 cc. of stock solution of invertase to which

were added in the following order, 10 cc. of hydrochloric acid, citrate buffer to give the final solution a pH of 2.1, 5, 10, 15, 20, 25 cc. of the iron sol, and enough water to bring the volume up to 100 cc. The second series contained 50 cc. of stock invertase solution per 100 cc. of final solution and the third, 65 cc. These solutions were allowed to stand from 10 to 30 minutes with frequent gentle shaking. At the end of this time the solutions which showed precipitation were selected, the concentration of sol and invertase used, noted and filtered. The other solutions were discarded. 10 cc. of 0.1 M disodium citrate were added to each 25 cc. of filtrate thus obtained. These solutions were now dialyzed to remove all salts present and the normality of the enzyme tested as follows: After diluting the invertase solution to some convenient strength, 20 cc. of this solution were added to 200 cc. of sucrose solution, containing 22 gm. of sucrose and 22 cc. of 0.1 M citrate buffer.

If abnormality had not now been produced in any case, or only a slight decrease in the sucrose hydrolysis velocity constant, n , obtained, then the process was repeated from the beginning, varying to a greater extent the iron sol and invertase concentrations in the region where precipitation occurred. With one of the preparations used, namely invertase K, it was found that precipitation occurred only after standing several hours. Even after standing this length of time, the precipitation of the iron sol was not complete in any case. To overcome this difficulty, it was found that the addition of a drop of M sodium chloride to the solution with that concentration of the iron sol and invertase, which gave the greatest amount of precipitate, resulted in immediate and complete precipitation of the iron sol and left the invertase abnormal.

Abnormal invertase solutions prepared in this manner gave no test for iron with potassium ferrocyanide, either before or after the addition of concentrated sulfuric acid.

The concentration of sol and invertase preparation used for the three preparations, above mentioned, are summarized in Table XI. In this table the magnitude of the effect in increasing the activity per unit of dry weight, by treating the invertase preparation with the iron sol, is also indicated.

Hydrolysis Procedure.—In general, the hydrolysis procedure was that in use in this laboratory, and outlined by Vosburgh (4).

A sucrose-buffer solution was made up in such a way at 25°C. that when a convenient number of cubic centimeters of invertase solution were added, the resulting hydrolysis mixture contained its components in the following concentrations.

Sucrose	10 gm. per 100 cc.
Buffer	0.01 molar.
Invertase.....	Such as to give a convenient rate of hydrolysis.

In order to determine the extent of hydrolysis at any time, a sample of the hydrolysis mixture was pipetted directly into a portion of 0.1 M sodium carbonate solution, which stopped the reaction. From 15 minutes to 2 hours after this, as recommended by Hudson (9), the optical rotations of these solutions were determined. The initial rotation of the hydrolysis mixture was determined by adding a proportionate amount of invertase and sugar solution to the sodium carbonate solution, in the order named. The final readings were taken 2 to 7 days after the experiment was performed.

Control and Measurement of the pH.—The pH of all experiments was fixed by the use of citrate buffers, mixtures of hydrochloric acid, and disodium citrate, according to Sørensen (10). Stock solutions of the particular mixtures required, were made up in 0.1 M concentration, and used in all experiments in 0.01 M concentration. The pH of all experiments, excepting the hydrolyses, was determined electrometrically with the bubbling hydrogen electrode against a saturated potassium chloride-calomel cell, joined by a saturated potassium chloride salt bridge. The pH of the hydrolyses was determined colorimetrically, using the necessary Clark and Lubs indicators (11). The citrate standards used, were calibrated electrometrically.

SUMMARY.

1. Of three normal invertase preparations tried, all could be made abnormal by treatment with ferric oxide hydrosol and simultaneously there was an increase in the activity per unit of dry weight.

2. Abnormal invertase, prepared by this method, was unstable at pH about 4.5 and 25°C., a fact observed by Nelson and Holander for other abnormal invertase preparations.

3. Abnormal invertase, prepared by this method, was restabilized by the addition of inactivated normal invertase preparation, gelatin, or egg albumen, but not by an inactivated abnormal invertase preparation.

4. Abnormal invertase, prepared by this method, can be restabilized in varying degree, depending on the concentration of added inactivated normal invertase preparation, and further this restabilization was reversible with dilution.

5. Normal invertase preparation, rendered unstable by dilution, was restabilized by the addition of gelatin, egg albumen, or inactivated normal invertase preparation.

6. At pH 2.2 and 25°C. abnormal invertase is less stable than normal invertase. The addition of inactivated normal invertase preparation caused an increase in the stability of the former, but a decrease in the stability of the latter, when present in large excess. The use of inactivated abnormal invertase preparation failed to produce either of these effects.

7. Inactivating invertase at pH 2.2 and 25°C. at different invertase preparation concentrations resulted in the more concentrated inactivating faster at first than the less concentrated, but slower in the latter part of the reaction.

8. A second type of abnormality has been produced; namely, one due to increasing activity.

9. The inactivation of more concentrated invertase preparation solutions at pH 2.2 and 25°C. was partially reversible with dilution and a change of the acidity of the solution to pH about 4.5. This effect was not observable with solutions less concentrated with respect to invertase preparation.

10. At pH 4.8 and at temperatures 60 and 65°C. normal invertase preparation is not more stable than abnormal. In this respect the relative stabilities of the two preparations differed from the relative stabilities observed at 25°C. and hydrogen ion concentrations, pH 2.2 and 4.8.

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STUDIES IN NUCLEIN METABOLISM.

II. THE ISOLATION OF A NUCLEOTIDE FROM HUMAN BLOOD.

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Indirect evidence was recently brought forward¹ to show that human blood contained appreciable quantities of purine bound as nucleotide. What appears to be a pentose nucleotide has since been isolated from human blood and its physical properties, elementary composition, and decomposition products have been studied.

Fresh whole blood in 500 cc. lots was freed from protein with picric acid. The filtrate was neutralized to Congo red and the precipitate resulting from the addition of a large excess of uranyl nitrate was centrifuged off, suspended in water, and brought into solution with the least possible amount of sulfuric acid. A strong solution of mercuric sulfate was then added and the resulting precipitate was washed and decomposed with ammonium sulfide. After boiling off the sulfide the inorganic phosphates were removed with magnesia mixture. The clear alkaline liquid was made acid with acetic acid and neutral lead acetate added in large excess. The lead precipitate was washed and decomposed with hydrogen sulfide. After removal of the excess sulfide the clear fluid was allowed to evaporate in a vacuum desiccator.

There remained behind a sticky, slightly yellow mass, very soluble in water and insoluble in alcohol. A watery solution of this mass showed the following properties.

1. It was strongly acid in reaction.
2. There was no evidence of free phosphate; none, that is, precipitable with alkaline magnesia mixture.

¹ Jackson, H., Jr., *J. Biol. Chem.*, 1923, LVII, 121.

3. On boiling with orcinol HCl there developed a strong blue-violet color.

4. On boiling with phloroglucin HCl there developed a deep cherry-red color. An amyl alcohol extract of the color showed an absorption band at the D line.

The gluc-like mass was subjected to prolonged rubbing with absolute alcohol and there resulted a dry, sandy white powder. The yield of this crude product averaged 100 mg. per liter of blood. This powder was purified through the neutral lead salt (in acid solution) and again dried with alcohol.

The following analyses were made.

1. Macro Kjeldahl showed the nitrogen percentage to be 14.40.

2. The total phosphorus content after digestion with sulfuric acid was 9.52 per cent.

3. A portion was subjected to mild acid hydrolysis (2.5 per cent sulfuric acid at 100°C. for 2½ hours). The phosphorus liberated by this treatment (purine phosphorus) was 4.64 per cent.

4. Another portion was subjected to mild acid hydrolysis with 2.5 per cent sulfuric acid for 2½ hours in a water bath. The mixture was made alkaline with ammonia and an excess of ammoniacal silver nitrate was added. There resulted a gelatinous precipitate insoluble in ammonia. This was washed with water until free from ammonia, decomposed with hydrogen sulfide, and after removal of the excess hydrogen sulfide and acidification a saturated solution of picric acid was added. A voluminous crystalline precipitate resulted (see Fig. 1), almost insoluble in water and easily soluble in alcohol. The crystals decomposed with evolution of gas at 276–280°C. A macro Kjeldahl showed the total nitrogen to be 29.3 per cent. The picrate was subjected to a preliminary reduction with pure zinc dust and an excess of concentrated hydrochloric acid for 1½ hours at 100°C. Concentrated sulfuric acid was then added and the rest of the determination was carried out as in the usual Kjeldahl method. (Adenine picrate, 30.8 per cent. (See Fig. 2.)) The yield of picrate was 52 per cent by weight of the original substance. "Adenine uracil nucleotide" should yield 54 per cent by weight of adenine picrate.

A portion of the picrate was subjected to the process for the recovery of adenine from adenine picrate. A white, amorphous



FIG. 1 Adenine picrate from human blood. Rapid crystallization from a drop of solution on a cover slip. $\times 250$.

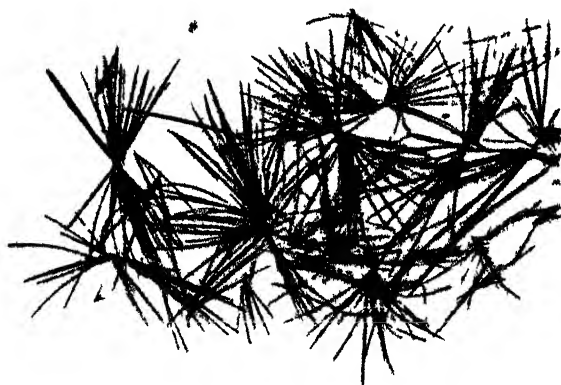


FIG. 2 Adenine picrate from yeast. Same conditions of crystallization as in Fig. 1. $\times 250$.

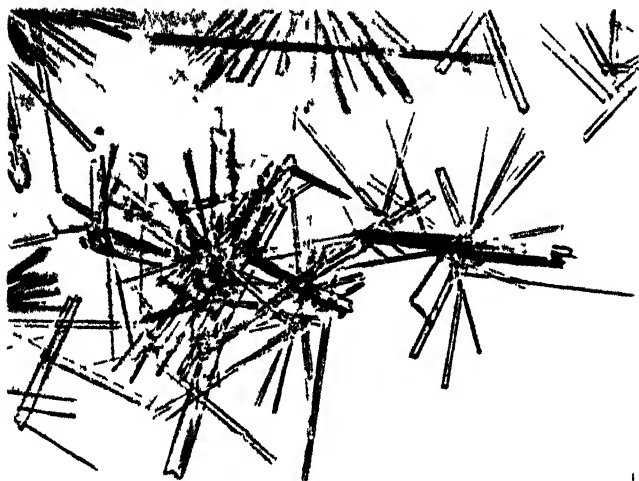


FIG. 3. Adenine sulfate from yeast. Rapid crystallization. $\times 250$.

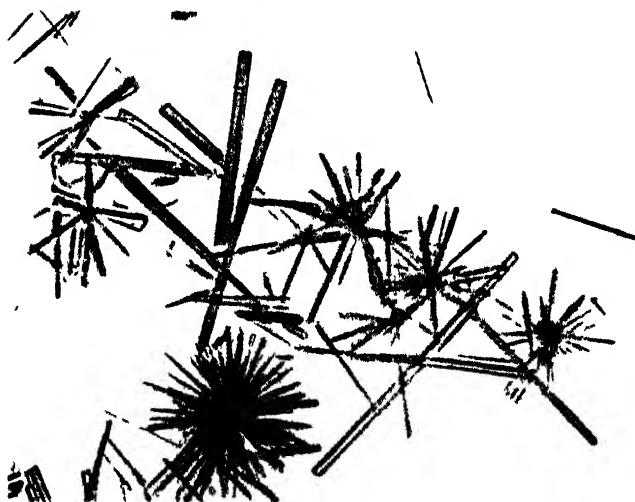


FIG. 4. Adenine sulfate from human blood. Same conditions of crystallization as above. $\times 250$.

powder was recovered. It gave only the faintest yellow color on evaporation with nitric acid. Picric acid produced a precipitate of very fine, pale yellow needles in a solution of this powder when the concentration of the latter was 1 part in 10,500. The powder was crystallized out of sulfuric acid and the resulting crystals were recrystallized out of water. (See Fig. 4.) For comparison, adenine sulfate from yeast, crystallized under the same conditions, is shown in Fig. 3. Micro analysis of the crystals recovered from the picrate showed the nitrogen content to be 33.8 per cent. Adenine sulfate; nitrogen 34.8 per cent.

Thus there has been isolated from human blood a substance precipitable with lead acetate in acid solution, soluble in water and insoluble in alcohol and giving a strong pentose reaction. It proved to contain 14.40 per cent nitrogen, 9.4 per cent total phosphorus, and 4.68 per cent hydrolyzable phosphorus. It yielded a picrate which showed the analysis, melting point, and crystalline structure of adenine picrate. This picrate in turn yielded a substance giving the approximate analysis for adenine sulfate and crystallizing in the same form and manner as that substance. The combined phosphoric acid, pentose, and nitrogenous substance indicate a nucleotide from plant nucleic acid and not from hexose nucleic acid. These figures correspond rather closely with the analysis and decomposition of the so called adenine uracil nucleotide.

The fact that approximately one-half the phosphorus is liberated by mild hydrolysis is in accordance with Jones' findings that a mixture of a purine and a pyrimidine nucleotide (originally supposed to be a purine-pyrimidine nucleotide) gives off one-half its phosphoric acid easily and holds the other half firmly. The above analytical findings suggest, therefore, that the substance under consideration was a mixture or combination of equal parts of a purine and a pyrimidine nucleotide. The nucleotide leakage is proved by the isolation of adenine as well as phosphoric acid after mild acid hydrolysis of the substance isolated.

In a previous paper¹ the belief was expressed that adenine nucleotide formed the major part of the nucleotide content of human blood. At that time no nucleotide had been isolated. The substance isolated and analyzed above is either the so called

adenine uracil dinucleotide or what is far more likely, a mixture of nucleotides; for there is but scant evidence that dinucleotides actually exist.

CONCLUSION.

A substance has been isolated from human blood which is believed to be adenine nucleotide combined with or mixed with an equal quantity of a pyrimidine nucleotide.

THE EFFECT OF ETHER ADDED IN VITRO ON THE CARBON DIOXIDE AND CHLORIDE DISTRIBUTION BETWEEN CELLS AND SERUM.*

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In a recent study Van Slyke, Wu, and McLean (1923) have demonstrated the importance of certain factors in controlling the electrolyte and water distribution in the blood. It is our intention to apply later the method which they have outlined to the study of the disturbance of acid-base equilibrium which occurs in ether anesthesia (Van Slyke, Austin, and Cullen, 1922; Cullen, Austin, Kornblum, and Robinson, 1923). It seemed not impossible, however, that ether might significantly alter the behavior of the blood cell membrane in its relation to the electrolytes and thus disturb the equilibrium relations we proposed to study. We accordingly carried out the following experiments to test the effect of ether added *in vitro* to the blood upon the CO₂ capacity and pH of the blood and upon the distribution of CO₂ and chloride between cells and serum.

Method.

Blood was taken from the left ventricle of a dog into tubes under paraffin oil and defibrinated by a technique (developed by Gram) which causes no visible hemolysis in dog's blood and which removes a relatively small number of cells in the fibrin.

Hemolysis when it occurs during defibrination is usually caused by the formation of loose, unattached clots containing a

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large amount of corpuscles, which are rubbed and squeezed against the wall. The technique described is designed to prevent this. The blood is taken in a cylindrical vessel, so that the stirring rod has free access to all parts of the blood. It is stirred by a gentle circular movement of the rod and not beaten (about one revolution a second). The stirring is done with a glass rod, the lower end of which has been heated and slightly expanded by pressing it against a metal plate. This dilatation prevents the fibrin from slipping off and causes it to wind itself closely around the rod when this is moved circularly in the same direction. Also it prevents the entire length of the rod from being rubbed against the wall of the vessel.

It is important that the defibrination should begin before any clotting with formation of loose coagula has taken place. In a great number of defibrinations on dog's blood carried out by this technique we have not had a single instance of visible hemolysis.

The defibrinated blood was pooled under oil and sodium fluoride added to make 0.1 per cent. We observed as did Evans (1922) that without sodium fluoride there was sometimes a tendency of the CO₂ capacity of the whole blood to decrease on standing. The blood was then divided into two portions. The first portion was saturated in two successive tonometers prepared according to the "second saturation method" of Austin, Cullen, Hastings, McLean, Peters, and Van Slyke (1922). The tonometers were prepared to give an atmospheric CO₂ concentration of 1.00 mm. The blood was saturated in each tonometer in a water bath at about room temperature for 10 minutes. The second portion of blood was similarly saturated in two tonometers similarly prepared to give an atmospheric CO₂ tension of 1.00 mm but into the first of which sufficient ether had been introduced to bring the blood to an ether content of about 0.13 gm. per 100 cc. and into the second of which sufficient ether had been introduced to maintain this concentration in the blood. The distribution coefficient for ether was taken from the curve of Shaffer and Ronzoni (1923) and the concentration obtained is comparable with that shown by White (1923) and by Ronzoni (1923) to be present in a fully anesthetized animal. After the second saturation the blood was run under oil into a centrifuge bottle, filling

the bottle, into a tube under oil for CO₂ analysis, and into a small flask for hematocrit and chloride determination. The centrifuge bottles were stoppered and centrifuged and the serum was removed under oil for CO₂ analysis, colorimetric pH, and chloride analysis.

Analytical Methods.

CO₂ analyses were performed with the constant volume apparatus of Van Slyke (1921) using the modified technique developed by Austin (1924) for serum and blood containing ether. Chloride analyses were done by the method of Van Slyke (1923). Cell volumes were measured by the hematocrit method, centrifuged until transparent, and performed in triplicate or quadruplicate. pH was read by Cullen's colorimetric method (1922) and the values were expressed as the colorimetric pH of the diluted serum at 20°C. All analyses were done in duplicate or triplicate except the pH determinations of Experiment 9.

RESULTS.

The results of two experiments are given in Table I. Several previous experiments during which the technique finally adopted was being developed are consistent with these.

The CO₂ capacity of blood and serum is identical, with and without ether, and so also is the distribution of CO₂ between cells and serum. The chloride distribution between cells and serum and the pH are identical, with and without ether.

The data of these experiments permit a comparison of the distribution of chlorides and of CO₂ in cells and serum, respectively. Van Slyke, Wu, and McLean found that

$$\frac{\frac{\text{Cl}_i}{\text{H}_2\text{O}_i}}{\frac{\text{Cl}_e}{\text{H}_2\text{O}_e}} = \frac{\frac{\text{CO}_{2i}}{\text{H}_2\text{O}_i}}{\frac{\text{CO}_{2e}}{\text{H}_2\text{O}_e}}$$

TABLE I.

Analysis of blood and serum from two dogs after double equilibration, without and with ether (0.13 gm. per 100 cc.) in atmosphere of air with 1.00 mm CO₂ at temperatures of 19.5°C. in No. 9 and 21.2°C. in No. 10.

Experiment No.	Ether.	Blood Cl.		Blood CO ₂ .		Serum Cl.		Serum CO ₂ .		Serum H ₂ CO ₃ .		Serum BHCO ₃ .		Log $\frac{H_2CO_3}{BHCO_3}$	Colorimetric pH of diluted serum at 20°C.	Cell volume.	Cell Cl.	Cell CO ₂ .	(CO ₂) _s	(Cl) _s	(CO ₂) _s	(Cl) _s
		mm.	(Cl) _b	mm.	(CO ₂) _b	mm.	(Cl) _s	mm.	(CO ₂) _s	mm.	(H ₂ CO ₃) _s	mm.	(BHCO ₃) _s									
9	Without.	86.8		18.32		110.1		21.79		0.93		20.86		1.35	7.61*	46.0	59.4	14.25	1.53	1.85	1.19	1.27
	With.	86.8				109.8		21.81		0.93		20.88		1.35	7.64*	46.0	59.8			1.84		1.27
10	Without.	84.3		20.95		108.6		25.24		0.89		24.35		1.44	7.62	45.5	55.2	15.82	1.59	1.97	1.20	1.29
	With.	84.3		21.03		108.8		25.21		0.89		24.32		1.44	7.62	44.9	54.1	15.90	1.59	2.01	1.20	1.29

* Single determination.

When this relation holds the following are also true

$$\frac{\frac{\text{Cl}_s}{V_s}}{\frac{\text{Cl}_c}{V_c}} = \frac{\frac{\text{CO}_{2s}}{V_s}}{\frac{\text{CO}_{2c}}{V_c}}$$

or

$$\frac{(\text{Cl})_s}{(\text{Cl})_b} = \frac{(\text{CO}_2)_s}{(\text{CO}_2)_b}$$

In which

$\frac{\text{Cl}_s}{\text{H}_2\text{O}_s}$ = concentration of serum chloride per kilo of serum water.

$\frac{\text{Cl}_c}{\text{H}_2\text{O}_c}$ = concentration of cell chloride per kilo of cell water.

$\frac{\text{Cl}_s}{V_s}$ = $(\text{Cl})_s$ = concentration of serum chloride per liter of serum.

$\frac{\text{Cl}_c}{V_c}$ = $(\text{Cl})_c$ = concentration of cell chloride per liter of cells.

$\frac{\text{Cl}_b}{V_b}$ = $(\text{Cl})_b$ = concentration of blood chloride per liter of blood.

and similarly for CO_2 .

Any difference between the ratios

$$\frac{\frac{\text{Cl}_s}{\text{H}_2\text{O}_s}}{\frac{\text{Cl}_c}{\text{H}_2\text{O}_c}}$$

and

$$\frac{\frac{\text{CO}_{2s}}{\text{H}_2\text{O}_s}}{\frac{\text{CO}_{2c}}{\text{H}_2\text{O}_c}}$$

will be associated with an equal percentile difference between the ratios $\frac{(\text{Cl})_s}{(\text{Cl})_c}$ and $\frac{(\text{CO}_2)_s}{(\text{CO}_2)_c}$, but there will be a smaller percentile

difference between the ratios $\frac{(\text{Cl})_s}{(\text{Cl})_b}$ and $\frac{(\text{CO}_2)_s}{(\text{CO}_2)_b}$. Not only in these two experiments but also in ten others on normal dogs have we consistently observed that the ratio $\frac{(\text{Cl})_s}{(\text{Cl})_b}$ exceeds by about 6 per cent the ratio $\frac{(\text{CO}_2)_s}{(\text{CO}_2)_b}$. This is shown in Table II. We can give

TABLE II.

Showing Relation of Chloride and Carbon Dioxide Distribution in Serum and Whole Blood in Repeated Bleedings on Three Normal Dogs.

Experiment No.	$\frac{(\text{Cl})_s}{(\text{Cl})_b}$	$\frac{(\text{CO}_2)_s}{(\text{CO}_2)_b}$	$\frac{\text{Ratio Cl}}{\text{Ratio CO}_2}$
4	1.24	1.28	0.97
	1.21	1.19	1.02
1	1.24	1.20	1.03
	1.32	1.24	1.06
5	1.27	1.23	1.03
	1.23	1.12	1.10
39	1.27	1.22	1.04
	1.27	1.18	1.08
38	1.27	1.21	1.05
	1.25	1.13	1.11
40	1.30	1.23	1.06
	1.32	1.19	1.11
8	1.26	1.18	1.07
2	1.27	1.19	1.07
9	1.27	1.19	1.07
10	1.29	1.20	1.07
	1.29	1.20	1.07
Average.....	1.269	1.199	1.059

no explanation at present for this apparent divergence from the equality which was observed by Van Slyke, Wu, and McLean and which would seem to be demanded by Donnan's Law of membrane equilibrium, if HCO_3^- and Cl^- are distributed simply in the water of the cell and serum, respectively, as suggested by Van Slyke, Wu, and McLean.

CONCLUSIONS.

1. The addition of ether to blood *in vitro* in a concentration comparable with that present in the blood of a fully anesthetized animal causes no change in the CO₂ or chloride content of cells or serum, nor in the pH, nor in the cell volume when the blood is equilibrated at a given CO₂ tension.

2. With or without ether present we have consistently observed in dogs' blood that the ratio $\frac{(\text{Cl})_a}{(\text{Cl})_b}$ is about 6 per cent higher than the ratio $\frac{(\text{CO}_2)_a}{(\text{CO}_2)_b}$.

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THE FATTY ACIDS OF BLOOD PLASMA.

II. THE DISTRIBUTION OF THE UNSATURATED ACIDS.

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According to Leathes' hypothesis of fat catabolism, the fatty acids of the fat of the food and bodily stores are to be regarded from a metabolic point of view as "inactive" and they become "active" by a process of desaturation—removal of hydrogen with the production of double bonds—a change which renders them more susceptible to further oxidation and possibly to other changes. The desaturation is believed to take place in the liver and the desaturated acids are carried by the blood to the tissues for utilization. We should expect, therefore, to find in the blood a considerable proportion of fatty acids more unsaturated than oleic acid which is the most important and frequently the only unsaturated acid of the food and stored fat. In recent work (1) it has been possible to demonstrate in blood plasma fatty acids of a considerably higher degree of unsaturation than oleic acid and forming a relatively large fraction of the fatty acids of the plasma lipoids. The liquid (unsaturated) fatty acids were found to compose two-thirds or more of the total fatty acids and to have average iodine numbers varying from 118 in the sheep to 133 in the pig, 147 in beef plasma and 155 in dog plasma. The distribution of these fatty acids, *i.e.* the form of combination in which they are carried in the blood, becomes then a matter of interest and the following work was undertaken in order to obtain some information on this point.

The known forms of fatty acid combination in fasting blood are phospholipoids, of which the best known are the lecithins and the closely related cephalins, the cholesterol esters of the fatty acids—according to Hurthle (2) mainly palmitic and oleic

acids—and probably also some fat although the presence of the latter has never actually been demonstrated. The procedure was planned so as to bring about an approximate separation of these groups so that their content of unsaturated fatty acid could be examined separately.

Procedure.

Samples of blood plasma, generally of about 1,000 cc. each, were mixed with 2 volumes of 95 per cent alcohol and allowed to stand overnight. The precipitated proteins were filtered on a Buchner funnel and completely extracted with hot alcohol in a continuous extractor designed by Clarke (3), the extraction taking about 8 hours. At first the whole volume of alcohol needed for the extraction was used throughout the extraction, but the procedure was later modified by using several small portions of alcohol so that each portion with its contained lipid was not exposed to the boiling temperature for more than 2 hours, the purpose being to avoid as far as possible decomposition of the more unstable lipoids by boiling. The combined extracts were evaporated to dryness at about 45°C. in partial vacuum. The first filtrate from the protein was similarly evaporated at low temperature to about one-third of its volume, at which point it foamed so much that further evaporation was difficult. The solution was, therefore, treated with about one-third its weight of solid ammonium sulfate, after which evaporation was continued until the alcohol was gone, then the solution was extracted by shaking out with ether. The same ether was used to dissolve the fatty material from the dried residue of the main alcoholic extract and the ethereal solution after filtration was evaporated nearly to dryness. At first the drying of this and similar material further along in the procedure was carried out quickly in air, but later a stream of carbon dioxide was used so as to minimize oxidation, although without notable effect on the iodine numbers of the various fractions.

Throughout the work, even when carbon dioxide was used, every effort was made to carry the processes through as quickly as possible, avoiding exposure to light and contact of the material with air, especially when not covered with solvent, and wherever a delay was unavoidable keeping the material under solvent in a stoppered container in the cold (5°C.).

Hot extraction with alcohol, as carried out above, was found to be very effective. In testing the completeness of the extraction the extracted protein residues were digested with strong (20 per cent) alkali for 8 hours, then acidified, the mixture was thoroughly extracted with ether, the ether evaporated, and the residue dried and extracted with pentane. The material in the pentane, after evaporation and drying, was weighed. In no case was more than 50 mg. of fatty material obtained.

Fractionation of the dry total lipid was carried out as follows:

Lecithin Fraction.—The total lipid was dissolved in a small amount of dry ether and the lecithin fraction precipitated by the addition of excess of acetone. The first precipitate was dissolved in dry ether and precipitated a second time, then after evaporation of the acetone it was saponified by heating with alcoholic sodium hydroxide for 4 hours, after which the fatty acids were liberated by acidification with hydrochloric acid and extracted with pentane. As a routine they were put through the procedure described below for separation from unsaponifiable matter. The amount obtained was generally negligible, but occasionally enough was found to justify the procedure. The fatty acids were dried in a current of carbon dioxide, weighed, and an iodine number determination was made. The amounts of fatty acid recovered were in general much too small to account for the amounts of phospholipoids ordinarily believed to be present in plasma. The explanation of the deficiency probably lies in the fact that insoluble residues are formed at certain points in the procedure which probably contained the altered phospholipoid; also, that in carrying out the various processes some of the phospholipoid was probably decomposed. The fact that a considerable amount of free fatty acid (see below) was always present in the lipoidal material which would hardly have existed in the plasma itself, bears out the latter explanation.

Fractionation of the Lecithin-Free Residue.—Various solvents—acetone, ethyl acetate, methyl alcohol, ether with ethyl alcohol, and ethyl alcohol alone—were tried as fractioning agents and the most generally suitable one was found to be ethyl alcohol. In the fractionation with alcohol enough solvent was added just to dissolve the lipid residue at the boiling point, the solvent allowed to cool and to stand in a stoppered flask in a dark place

until the next day. The separated material was reprecipitated in the same way and the residual solvent added to the first portion. The separated fraction was saponified by boiling for 8 hours with sodium ethylate with a return condenser, after which the alcohol was boiled off, the residue taken up with water, acidified, and the combined fraction submitted to separation into fatty acid and unsaponifiable matter according to the Kuma-gawa-Suto procedure. The separated material was dried in a stream of carbon dioxide and weighed.

The second fraction was obtained by concentrating the mother liquor to about one-third its volume and again letting stand overnight. The separated material was reprecipitated and further treated as in Fraction I. In the first part of the work the third and later fractions were obtained by further concentration of the mother liquor, but when it was discovered that the material contained free fatty acid, the separation of this material was made after Fraction II, as described below. The residue after separation of the fatty acids was further fractioned by ethyl and later by methyl alcohol in the same way as the earlier alcohol fractions.

Iodine number determinations were made of the fatty acids by the Hanus method at once after separation. Controls were run on all solvents to detect iodine absorbing material but were found negative.

Fatty Acid Fraction.—Early in the course of the work it was found that the extracted material contained free fatty acid. An attempt was made to separate it as soap by titration in petroleum ether with alcoholic alkali, but so much of the soap remained in solution in the solvent that an accurate separation was not possible. Accordingly in later work the separation was made by the process used in separating unsaponifiable matter—solution in pentane, addition of 1 volume each of 0.1 N alcoholic potassium hydroxide and of water. The fatty acids passed as soaps into the watery alcohol from which they were recovered by acidification and extraction. The other lipid material remained in the pentane from which it was recovered by evaporation and was then further fractioned as above.

The data obtained by examination of the fractions are presented in Table I.

DISCUSSION.

Lecithin Fractions.—The composition of the lecithin fraction appeared to be about the same for all the animals examined so that all may be considered together.

As noted above the amount of fatty acid recovered by saponification of the lecithin fraction was much smaller than was to be expected from the amount of lecithin ordinarily believed to be present in the blood plasma of the animals examined. The reasons for the low values are probably as noted above—insolubility or decomposition of the lecithin during the processes of separation. The samples obtained are, however, believed to be representative since there is no definite difference in iodine-absorbing power between the large and small fractions and because the iodine values of the free fatty acids, which presumably result from the decomposition, are of the same order (although somewhat higher) as those obtained from the fatty acids of the lecithin fraction. Assuming that the samples are representative, the low iodine values of the fatty acids call for comment since they seem to indicate that the blood lecithins contained only fatty acids of a relatively low degree of unsaturation and that, therefore, lecithin was not important in the transport of unsaturated fatty acids from the liver. However, the fatty acids obtained are quite obviously mixtures of solid and liquid acids since, although solid at room temperature and therefore containing a considerable proportion of saturated acids, the material has a moderately high iodine number (averaging in beef 71, in pig 80, and in dog 89) which shows the presence of unsaturated and therefore liquid acids. It has rarely been possible to obtain enough material to make the separation into solid and liquid acids, and in the few cases where it was tried a satisfactory separation was not obtained so that it is not possible to speak definitely regarding the proportions of the two constituents, except that there was found in the fatty acids from the lecithin fraction and from the soap fraction a fairly large proportion of solid fatty acids with a melting point of about 50°C. Recent investigations by Levene and Simms (4) have indicated that the lecithins of the liver and brain are almost always mixed lecithins, each molecule of lecithin containing one molecule of solid fatty

TABLE I.

Lecithin fraction		Lecithin-free fractions from alcohol.															
		Fraction I.			Fraction II			Fraction III.			Fraction IV.			Other fractions.			
Blood sample.	Fatty acids.	Iodine numbers.	Unsaponifiable.	Fatty acids.	Iodine numbers.	Unsaponifiable.	Fatty acids.	Iodine numbers.	Unsaponifiable.	Fatty acids.	Iodine numbers.	Unsaponifiable.	Fatty acids.	Iodine numbers.	Unsaponifiable.	Fatty acids.	Iodine numbers.
			Beef.														
cc.			0.387	0.317 (0.282)	169	0.076	0.047 (0.055)	156	0.10	0.171	89						
500			0.120	0.090 (0.087)	96	0.366	0.272 (0.255)	149	0.346	0.437	114						
670	0.096	80	0.816	0.574 (0.592)	130	0.110	0.089 (0.080)	154	0.096	0.178	109						
1,000	0.110	87	0.227	0.168 (0.165)	154	0.092	0.076 (0.067)	143	0.060	0.089	100						
500	0.094	88	0.292	0.211 (0.213)	120												
1,400			0.114	0.112	150	0.087	0.071 (0.63)		0.057	0.104							
500			0.663	0.550 (0.484)	128	0.143	0.123 (0.103)	118	0.142	0.278	93						
1,200	0.131	85	0.457	0.318 (0.333)	130	0.128	0.095 (0.092)		0.170	0.288							
1,100			1.120	0.862 (0.818)	133	0.129	0.097 (0.092)	102	0.070	0.217	115						
750																	

970	0.169	84	1.083	0.720 (0.788)	151	0.111	0.044	124	0.291	0.769	128						
2,000	0.184	80	1.464	1.078 (1.070)	158	0.120	0.090 (0.087)	164	0.696	1.490	120						
2,880	2.30	81	3.268	2.976 (2.40)	135	1.295	0.223	141	0.454	2.102	110	0.591	0.592	102	*	0.913	97
2,100	0.177	39	2.78	2.05 (2.03)	140	0.251	0.267	125	0.251	0.739	123	0.393	0.772	106	*	0.947	85
970	0.198	44	0.44	0.33 (0.32)	112	0.164	0.127 (0.118)	136	0.08	0.318	116						
750	0.158	54	0.29	0.21 (0.21)	145	0.121	0.097 (0.087)	108	0.122	0.118	118				*	0.182	105
1,000	0.021	60	0.336	0.246	164	0.142	0.103 (0.102)	190	0.072	0.450	111	0.181	0.095	149	*	0.45	90
Average ...		71			139			147			111			119			94

Fig.

800	0.243	38	0.131	0.182	0.967	1.064	132								*	0.263	57
1,700	0.851	92	1.271	1.251	0.313	0.215	170								*	0.167	47
1,700	2.102	65	1.202	0.876	0.301	0.227	106								*	0.547	69
1,900			1.257	1.335	0.252	0.520	93								*	0.211	96
1,000	0.222	91	0.411	0.350	0.103	0.08	141	0.102	0.097						*	0.140	99
1,000	0.200	95	0.410	0.372	0.223	0.233	119	0.124	0.103	117					*	0.122	99
1,400	0.264	83	0.549	0.574	0.273	0.282	151	0.219	0.350	91					*	0.350	98
1,100	0.203	98	0.687	0.875	0.199	0.077	95	0.078	0.284	121					*	0.468	97
1,130	0.182	89	0.922	0.723	0.207	0.100	103	0.034	0.127	128					*	0.453	95
1,000			0.639	0.399	0.179	0.092	107	0.053	0.180	121					*	0.688	98
1,270	0.169	72	0.784	0.671	0.233	0.142	123	0.053	0.209	127					*		
Average ...		80					126			117							86

* Free fatty acid.

TABLE I—Concluded.

Lecithin-free fractions from alcohol.																
Lecithin fraction.		Fraction I.			Fraction II.			Fraction III.			Fraction IV.			Other fractions.		
Iodine numbers.		Fatty acids.		Unsaponifiable.	Fatty acids.		Unsaponifiable.	Fatty acids.		Unsaponifiable.	Fatty acids.		Unsaponifiable.	Fatty acids.		Iodine numbers.

acids to one of liquid acid. If the blood lecithins have their origin in the liver we would expect the same to be the case for them and, therefore, the iodine numbers of the unsaturated acids present would be about double those found. If this were the case the values would be of the same order as those of the unsaturated acids of liver lecithin which Levene found to be about 154. Of course, if the lecithins are, as is now believed, a universal means of transport of the fatty acids we should also expect that a certain proportion of the lecithins in the blood in fasting would contain fatty acids from the fat of the bodily stores and, therefore, of a lower iodine number than those originating in the liver. Until further work has given more exact information with regard to blood lecithin it can only be said that participation of lecithin in the transport of the unsaturated acids from the liver is not excluded by the present work—is probable but not proved.

The iodine values of the various samples of the lecithin fraction are mostly close to the average values noted, but there are occasional low values which are not to be explained by differences due to method and which indicate that the blood lecithin is probably not at all times uniform in composition.

The Alcohol Fractions.

Beef Plasma.—In Fractions I and II the ratio of cholesterol to fatty acid is in almost all cases close to that required by the formula of cholesterol linolate as shown by the calculated values for the fatty acids which are placed in parentheses and directly below the fatty acid values actually found. As to whether the material is actually cholesterol linolate the following should be considered: (a) that the iodine numbers of the fatty acids found are considerably below those of linolic acid ($C_{18}H_{32}O_2$) which has an iodine number of about 180; and (b) that the fatty acids as separated contain some solid fatty acids. Separations of solid and liquid fatty acids on a number of samples have shown that there is ordinarily about 10 per cent of solid acids in the mixture with a melting point of about 55° . The presence of these solid acids would lower the iodine number of the mixture so that the values of the liquid acids would be somewhat higher than those found, but not as high as is required for linolic acid. The lower values

found may, of course, have been due to oxidation during the preparation, but considerable care was taken to avoid oxidation, especially in the later samples, and the author is inclined to think that the numbers found represent essentially those of the acids in the blood. Since the present investigation was largely exploratory—to determine the approximate distribution of the unsaturated acids—no special attempt was made to purify the fractions beyond a second crystallization and the fractions obtained quite evidently do not consist of a single substance. Reference may be made again to Levene and Simms' (4) study of the liquid fatty acids of liver lecithin which they found to have an iodine number of 154 and which were found to consist of oleic and arachidonic acids in the proportion of 1.3 parts of oleic to 1 of arachidonic, and to the work of Hartley (5), which is referred to by Levene, in which the presence of arachidonic acids in the liver was shown to be probable. If the iodine values of the fatty acids of the first two fractions be corrected for the solid acids as found, they would come very close to those reported by Levene for liver lecithin, which makes it possible that the acids bound to cholesterol in blood plasma are the same as those combined as lecithin in the liver. However, Hürthle's work on the cholesterol esters (see below) would seem to exclude any acids higher than C_{18} .

For purposes of comparison a few separations of cholesterol "oleate" were made by Hürthle's method (2), using 500 cc. of beef plasma. Analyses of these samples yielded the following results.

The analytical values are in general very similar to those given in Table I for Fractions I and II of beef blood and indicate that the same substances were obtained in both cases.

The amounts of cholesterol and fatty acid obtained from the alcoholic fractions from Table I agree rather better with the formula of cholesterol linolate than those of Table II and are also of more even composition. One important point which is clearly brought out by both series of analyses is that the substance which Hürthle called cholesterol oleate is probably not entirely or even mainly oleate but either linolate or a mixture of oleate with esters of fatty acids still more unsaturated. There can be no reflection on the work of Hürthle which was obviously

carefully done, but he did not have the advantage of the information supplied by iodine number determinations and the method of organic analysis is not sensitive enough to show a difference of two or even four hydrogen atoms in a molecule the size of oleic acid. On the other hand, the method would show differences of one and certainly two carbon atoms and as the values for carbon which he obtained are quite close to that required for oleic acid it is likely that the fatty acid with which he had to deal and, hence, also the one present in Fractions I and II of the present work, was an eighteen-carbon acid and, therefore, either linolic acid or a mixture of oleic with other less saturated eighteen-carbon acids.

TABLE II

Sample No	Cholesterol	Fatty acids	Iodine number.
I	0 417	0 301 (0 302)	145
II	0 180	0 165 (0 131)	133
III	0 180	0 103 (0 101)	161
IV	0 169	0 135 (0 123)	120
V	0 305	0 202 (0 220)	137

The fatty acids of the later fractions of beef plasma compose a much larger proportion of the fractions in the first two, in most cases two-thirds of the fraction and in many samples still more. There is no possibility that the fatty acids in these fractions are all combined with cholesterol and it is probable that the fraction contains a large proportion of fat. The lower iodine numbers of the fatty acids might be taken as evidence that the fatty acids of the fat were of a relatively low iodine value especially if the cholesterol ester present were the same as in the first two fractions. But the work of Hürthle (2) has shown that there is also some cholesterol palmitate present in plasma and this substance

would separate with the later fractions. Since no method has been found by which the fat can be separated out alone nothing definite can be said regarding the nature of the fatty acids combined in the fat of the plasma. The free fatty acid fraction was separated between alcohol Fractions II and III so that these fatty acids are not included in Fraction III or later fractions.

Pig and Dog Plasma.—The alcohol fractions of pig and dog plasma all contain a higher percentage of fatty acid than corresponding fractions of beef blood and much too high to correspond with the composition of cholesterol linolate. Their iodine numbers are lower throughout than in corresponding fractions of beef blood. If the cholesterol esters are of the same nature in the plasma of these animals as in beef plasma these facts may be interpreted to mean that there is present a considerable admixture of fat of which the fatty acids are of a relatively high iodine value. Cholesterol palmitate, as noted above, would appear in the later fractions but probably not to any great extent in the first two. The presence of a highly unsaturated fatty acid such as arachidonic ($C_{20}H_{32}O_2$), which Levene and, previously, Hartley have found in liver, is apparently excluded by the low iodine values found and by the work of Hürthle mentioned above, although a C_{20} acid would give a better agreement between the theoretical and the actual fatty acid values reported in the table.

The "unsaponifiable matter" from Fraction I and II in all cases is well crystallized and of light color—yellowish in the beef and nearly white in the others. It is probably largely cholesterol since when compared with a standard cholesterol by the Liebermann-Burchard color reaction it gives 70 to 90 per cent of the theoretical color value. Also a few crystallizations from alcohol give the characteristic cholesterol crystals which are further identified by melting point and optical properties.

The unsaponifiable matter from the later fractions is generally non-crystalline, sometimes liquid, and of a brown color with much lower color values by the Liebermann-Burchard reaction than that from the earlier fractions. Cholesterol can be obtained by crystallization from alcohol, but there is present another material, a brown, thick liquid of unknown composition.

The Free Fatty Acids.—As noted above, the iodine values of this fraction are throughout closer to those of the lecithin fraction

(generally a little higher) than to those of the alcohol fractions which indicates that they originate by decomposition of the lecithin. The amounts vary greatly and bear no relation to the amount of total lipid. The melting point of the mixture where determined was found to be about 45°C.

Corpuscles.—A few examinations were made of the fatty acid distribution in the lipoids of the corpuscles. For this purpose the corpuscles were washed once with normal salt solution, then hemolyzed by mixture with 1 volume of distilled water, saturated with ether, and the mixture was poured into 2 volumes of alcohol. Afterwards, the treatment and fractionation was the same as for plasma.

The iodine values of the lecithin fraction (see Table I) were of the same order as for the similar fraction of the plasma, indicating that the lecithin of the corpuscles is the same as that of plasma.

The alcohol fractions consist almost entirely of cholesterol, indicating that there is little if any cholesterol ester in the corpuscles, which is in agreement with the prevailing belief on this point.

In general the data presented show that a relatively large proportion of the unsaturated fatty acids are in combination with cholesterol and a much smaller proportion combined as phospholipoid. Owing to the chemical inertness of the cholesterol esters it is difficult to conceive of them taking the active part in fatty acid metabolism which this finding would seem to indicate. The presence of a cholesterase of which the function is the hydrolysis of these esters has been claimed, but very little is known about it. The facts presented, however, indicate that cholesterol must be taken into account in the consideration of the utilization of the unsaturated fatty acids. Its importance in general fat metabolism has been discussed in a previous communication (1) and the data in the present work bring it one step nearer to actual participation in the metabolism of the fatty acids.

The incompleteness of the data on the lecithin fraction makes it difficult to give a definite interpretation of the findings. If the assumption is correct that the blood lecithin is a mixed lecithin, containing one saturated and one unsaturated acid, the unsaturated acids would be of the same kind as those found in

the liver and a relationship between the liver lecithin and the blood lecithin would be indicated. Evidence as discussed above renders this assumption probable and, therefore, makes it reasonable to believe that lecithin is concerned in the transport of the unsaturated fatty acids in the blood.

SUMMARY.

The unsaturated acids of blood plasma are found to be largely in combination with cholesterol. The inference that the unsaturated acids are mainly transported from the liver to the tissues as cholesterol esters and that cholesterol, therefore, takes an active part in the metabolism of the fatty acids is difficult to accept because of the chemical inertness of the cholesterol esters. Nevertheless, the data presented give further support to the growing belief that cholesterol must be seriously reckoned with in the consideration of fat metabolism.

The data on the lecithin fraction are incomplete and leave open the question as to the part played by the phospholipoids in the transport and further metabolism of the unsaturated fatty acids, although its participation in these processes is shown to be probable.

The cholesterol esters of blood plasma consist not only of palmitate and oleate as shown by Hürthle but also of a large proportion of the esters of the more unsaturated acids.

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THE METABOLISM OF SULFUR.

VII. THE OXIDATION OF SOME SULFUR COMPOUNDS RELATED TO CYSTINE IN THE ANIMAL ORGANISM.*

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In previous studies (1, 2, 3) one of us (L.) has reported experiments which are concerned with the oxidation of sulfur in the cystine molecule. It was demonstrated that if deamination of cystine be prevented by "blocking" the amino group (as in phenyluraminocystine (1) or dibenzoylcystine (3)), the sulfur of the molecule was not oxidized normally and was excreted in the urine in large part in the unoxidized sulfur fraction. However, if normal oxidation of the sulfur was prevented in this way, it was shown that the cystine was converted to cysteine and a considerable amount of the cystine derivative administered appeared in the urine as a derivative of cysteine (2, 3). Recently Sherwin and his collaborators (4, 5) have reported similar results which in general confirm our earlier experiments. They also showed that if a derivative of cysteine with the amino group protected from deamination was fed to a rabbit, the cysteine was in part converted to cystine. Our own results and those of Sherwin are in accord with the current theory of the ready reversibility of the reaction $\text{cysteine} \rightleftharpoons \text{cystine}$.

Many other investigations which are related to the oxidation of various types of sulfur linkages in organic combination have been reported. Inasmuch as this present study is concerned only with the oxidation of mercapto or sulfide sulfur of the type similar to that naturally occurring in the protein molecule, a complete

* An abstract of a thesis submitted by R. M. Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Graduate School of the University of Illinois.

review of the literature on the oxidation of organic sulfur compounds is not attempted. The discussion of the oxidation of cyclic compounds containing sulfur in the ring (*e.g.*, thiophene, thioindoxyl) is omitted entirely and will be discussed in another place by one of us (L.).

Investigators who have studied the oxidation of the sulfur of the mercapto groups in aliphatic compounds are generally agreed that sulfur of this type is oxidized to sulfates and excreted in the urine in this form to a considerable degree. The ready oxidation of the sulfur of cysteine is well known. Smith (6) fed small doses of the sodium salt of ethyl mercaptan to dogs and noted slight rises in the sulfate sulfur of the urine. In one experiment in which a total of about 1.6 gm. was fed to a 7.5 kilo dog over a period of 4 days, 55 per cent of the sulfur fed appeared as extra sulfur in the urine and of this 53.7 per cent was present as sulfate sulfur. In a second similar experiment in which 4.99 gm. were fed during a 5 day period, 47 per cent of the ingested sulfur appeared in the urine, and 37.5 per cent of this extra sulfur was present as sulfate sulfur. Ethyl mercaptan itself was somewhat more toxic than the sodium salt. Of 1.021 gm. fed in a single dose, slightly less than 25 per cent was eliminated in the urine, and of this 70 per cent was sulfate sulfur. The interpretation of these experiments is complicated by the fact that the mercaptan was always somewhat toxic and that an increased nitrogen elimination was usually noted. Smith (7) also reports one experiment in which 2 gm. of the ammonium salt of thioglycollic acid were fed daily to a dog for 2 days. A larger dose caused vomiting and other toxic symptoms. The sulfate sulfur excretion was somewhat increased, indicating a partial oxidation of the sulfur of the complex. Smith (8) also fed ethyl thiocarbamate (thiourethane), $\text{NH}_2\text{CO}\cdot\text{S}\cdot\text{C}_2\text{H}_5$, and ethyl thiocarbamate, $\text{NH}_2\text{CS}\cdot\text{O}\cdot\text{C}_2\text{H}_5$, to dogs. The latter was much more toxic than the former and little oxidation (?) of its sulfur was noted. The sulfur of ethyl thiocarbamate, however, was oxidized to sulfates to a considerable extent. It is possible that in this case hydrolysis to ethyl mercaptan may have occurred with subsequent oxidation of the latter. Freise (9) noted after the enteral administration of thiouramil to dogs that the sulfur of its mercapto group was partially (48.1 per cent of the total sulfur recovered in

the urine) oxidized, while after feeding γ -thiopseudouric acid, although only 36 per cent of the sulfur fed was eliminated as urinary sulfur, of this 86 per cent appeared in the sulfate sulfur fraction.

The oxidation of the sulfur of compounds containing the disulfide sulfur linkage, -S-S-, has not been studied except in the case of cystine. In his study, Smith (10) has noted that the sulfur of ethyl sulfide was eliminated very slowly and that no evidence of any oxidation was apparent. Bivalent sulfur, which replaces oxygen in carbonyl groups, is apparently not oxidized as illustrated in the case of thiourea (11), the thiohydantoins (12), and thio-pyrimidines (13). The partially oxidized sulfur of sulfonic acids and sulfones is resistant to further oxidation (7, 14).

Thus there are recorded studies of the behavior of compounds containing sulfur in the types of linkage, C-SH (mercaptan), C-S-C (sulfide), C-SO₂ OH (sulfonic acid), C-SO₂-C (sulfone), =C=S (thioamide), and C-S-S-C (disulfide). Of these only the first, C-SH, and such others as may be hydrolyzed (*e.g.* thiourethane) or reduced (cystine) to form this type are readily oxidized in the organism. This is the more remarkable, in view of the fact that some of the other more highly oxidized forms are intermediary products of the oxidation of the mercaptan group *in vitro*. The mercaptan group, less readily oxidized *in vitro*, is the most readily oxidized *in vivo*.

In the present investigation, we have undertaken a study of the oxidation of some sulfur compounds which have a closer relationship to cystine or cysteine than most of those previously studied in order to obtain a more complete picture of the conditions governing oxidation of sulfur in the animal body.

EXPERIMENTAL.

The rabbits used in the experiments were fed daily 150 cc. of milk, 10 gm. of sucrose, and 10 gm. of hay, with water *ad libitum*. The sucrose was added to increase the calorific value of the diet and the hay in order to provide roughage, since we have found repeatedly that animals on such a concentrated diet as milk and sugar are better nourished if a small amount of hay be added also. This may be a question of roughage or of some other factor. This standard diet was fed for at least 3 days before the collection of

the urine was begun in order to be certain that the animal was accustomed to the diet and to insure as uniform a urinary excretion as possible. The urine was then collected in 24 hour periods by squeezing out the bladder and samples for at least 3 normal days were obtained and analyzed. The substance under investigation was fed through a stomach tube or injected subcutaneously and changes in the distribution of sulfur in the urine were noted. An after period completed each experiment.

Total sulfur was determined by the Denis modification of Benedict's method, inorganic sulfate sulfur and total sulfate sulfur by the methods of Folin, conjugated sulfate sulfur and organic sulfur were obtained by difference, as is customary. Nitrogen determinations by the usual Kjeldahl method were carried out on all samples in order to afford a check on any changes in protein metabolism. An increase in protein metabolism or tissue breakdown due to the toxic action of the substance administered would increase the sulfur elimination and might make it appear that an oxidation of the substance under investigation had occurred, when this had not been the case. If an increase in the sulfur output were due to increased catabolism of protein, this should be accompanied by a corresponding increase in the output of total nitrogen.

The compounds under investigation, thioglycollic, thiolactic, and thiodiglycollic acids, were Kahlbaum preparations, the purity of which was checked by analysis of the sulfur content.

DISCUSSION.

Thiolactic Acid, $\text{CH}_3\text{-CH}(\text{SH})\text{-COOH}$. -The results of typical experiments¹ with thiolactic acid are detailed in Tables I and II. The animals remained bright, there was no loss of appetite, and no toxic effects were noted at any time. In the experiment recorded in Table I, approximately 90 per cent of the sulfur fed as thiolactic acid was eliminated as "extra" sulfur the 1st day,

¹ In the experiments with thiolactic acid as well as those with the other compounds studied, a few typical experiments only are reported. In all cases, these results were checked with other animals. In order to condense the data these additional experiments are omitted. They are, however, recorded in the original thesis on file in the Library of the University of Illinois.

and 56 per cent of this appeared as extra inorganic sulfate. In the two experiments of Table II, 78 per cent (oral administration) and 84 per cent (subcutaneous injection), respectively, of the sulfur administered, was eliminated as extra sulfur the 1st day and of this 71 and 61 per cent, respectively, were completely oxidized,

TABLE I.

Rabbit I. Male. Weight 2.5 kilos. Daily diet: 150 cc. of milk, 10 gm. of sucrose, and 10 gm. of hay. "

Day.	Total sulfur.	Inorganic sulfate sulfur.	Conjugated sulfate sulfur.	Organic sulfur.	Total nitrogen	Remarks.
	gm.	gm.	gm.	gm.	gm.	
1	0.0233	0.0151	0.0019	0.0063	0.589	
2	0.0242	0.0130	0.0049	0.0063	0.593	
3	0.0229	0.0102	0.0048	0.0079	0.603	
4	0.1986	0.1137	0.0088	0.0761	0.582	{ 0.658 gm. thiolactic acid as sodium salt <i>per</i> os. (S=0.1974 gm.)
5	0.0397	0.0233	0.0056	0.0108	0.613	
6	0.0217	0.0091	0.0066	0.0060	0.598	

TABLE II.

Rabbit O. Male. Weight 2.0 kilos. Daily diet: 150 cc. of milk, 10 gm. of sucrose, and 10 gm. of hay.

Day.	Total sulfur.	Inorganic sulfate sulfur.	Conjugated sulfate sulfur.	Organic sulfur.	Total nitrogen.	Remarks.
	gm.	gm.	gm.	gm.	gm.	
1	0.0434	0.0304	0.0011	0.0119	0.838	
2	0.0446	0.0304	0.0026	0.0116	0.833	
3	0.0452	0.0321	0.0024	0.0107	0.793	
4	0.1369	0.0960	0.0049	0.0360	0.878	{ 0.391 gm. thiolactic acid as sodium salt <i>per</i> os. (S= 0.1173 gm.)
5	0.0497	0.0334	0.0033	0.0130	0.847	
6	0.0523	0.0386	0.0044	0.0093	0.865	{ 0.405 gm. thiolactic acid as sodium salt by sub- cutaneous injection. (S=0.1215 gm.)
7	0.1547	0.0982	0.0048	0.0517	0.915	
8	0.0328	0.0211	0.0017	0.0100	0.585	
9	0.0529	0.0360	0.0036	0.0133	1.010	

as evidenced by the rise in sulfate sulfur excretion. The changes in the conjugated sulfate sulfur in the feeding experiments are very slight, but it should be noted that similar slight increases were noted in all the other feeding experiments with thiolactic acid not recorded here. The constancy of the nitrogen output

indicates that the changes in the inorganic sulfate are due entirely to oxidation of the sulfur of the thiolactic acid and not to any increased tissue catabolism.

Thioglycollic Acid, $\text{CH}_2\text{SH}\cdot\text{COOH}$.—In contrast to thiolactic acid, thioglycollic acid was found to be somewhat toxic. In the earlier experiments quantities of thioglycollic acid which were comparable to those of the thiolactic acid administered in experiments already discussed were fed. The animals died within a few hours after feeding. The urine in the bladders after death showed a strong nitroprusside reaction.² In subsequent studies, in which smaller doses were given, the rabbits exhibited a decided malaise after the administration of the thioglycollic acid. The smaller doses sometimes proved fatal especially when a previous dose had been given (*cf.* Table IV).

In spite of the toxicity of the thioglycollic acid, its sulfur was nearly as completely oxidized and as rapidly eliminated in the organism of the rabbit as was the sulfur of the non-toxic thiolactic acid (Tables III to V). Thus in Table V, 75 per cent of the sulfur of the thioglycollic acid fed was eliminated as "extra" sulfur the 1st day and of this 49 per cent was oxidized to sulfates. In the experiment recorded in Table III, in which a relatively large dose of thioglycollic acid was fed, it would appear that more "extra" sulfur was eliminated than could be accounted for by the compound fed. However, a marked increase in the total nitrogen elimination occurred, presumably occasioned by an increased tissue catabolism due to toxicity of the thioglycollic acid. Normally a fairly constant ratio exists between nitrogen and sulfur in the urine of rabbits. As a result of feeding the relatively toxic thioglycollic acid, a distinct rise in the output of nitrogen occurred. Because of this increased protein catabolism, it is not allowable, in computing the "extra" sulfur of the urine of the experimental days, to subtract the average total sulfur of the preliminary days from the total sulfur of the experimental period, since with the rise in nitrogen there is, presumably, a corresponding rise in the sulfur excretion, sulfur which does not have its

² With sodium nitroprusside and ammonia, compounds which contain the mercaptan group give an intense ruby-red color. This test can be applied to urine since creatinine does not give a positive reaction when ammonia is used as the alkali.

TABLE III.

Rabbit P. Male. Weight 2.1 kilos. Daily diet: 150 cc. of milk, 10 gm. of sucrose, and 10 gm. of hay.

Day.	Total sulfur.	Inorganic sulfate sulfur.	Conjugated sulfate sulfur.	Organic sulfur.	Total nitrogen.	Remarks.
	gm.	gm.	gm.	gm.	gm.	
1	0.0496	0.0326	0.0035	0.0135	0.785	
2	0.0407	0.0243	0.0036	0.0128	0.685	
3	0.0389	0.0229	0.0032	0.0128	0.650	{ 0.372 gm. thioglycollic acid as sodium salt per os. (S=0.13 gm.)
4	0.2079	0.1129	0.0072	0.0878	1.085	
5	0.0407	0.0268	0.0051	0.0088	0.995	
6	0.0444	0.0321	0.0035	0.0088	0.833	

TABLE IV.

Rabbit R. Male. Weight 2.1 kilos. Daily diet: 150 cc. of milk, 10 gm. of sucrose, and 10 gm. of hay.

Day.	Total sulfur.	Inorganic sulfate sulfur.	Conjugated sulfate sulfur.	Organic sulfur.	Total nitrogen.	Remarks.
	gm.	gm.	gm.	gm.	gm.	
1	0.0441	0.0280	0.0046	0.0115	1.325	
2	0.0452	0.0262	0.0066	0.0124	1.244	
3	0.0354	0.0190	0.0090	0.0074	1.127	{ 0.296 gm. thioglycollic acid as sodium salt by subcutaneous injection. (S=0.103 gm.)
4	0.1088	0.0545	0.0074	0.0469	1.200	
5	0.0511	0.0291	0.0069	0.0151	1.146	
6*	0.0305	0.0159	0.0040	0.0106	1.125	

*On the 7th day 0.330 gm. of thioglycollic acid as sodium salt given per os. The animal died in 4 hours.

TABLE V.

Rabbit I. Male. Weight 2.3 kilos. Daily diet: 150 cc. of milk, 10 gm. of sucrose, and 10 gm. of hay.

Day.	Total sulfur.	Inorganic sulfate sulfur.	Conjugated sulfate sulfur.	Organic sulfur.	Total nitrogen.	Remarks.
	gm.	gm.	gm.	gm.	gm.	
1	0.0240	0.0103	0.0063	0.0074	0.503	
2	0.0227	0.0091	0.0041	0.0095	0.578	
3	0.0206	0.0062	0.0071	0.0073	0.600	{ 0.205 gm. thioglycollic acid as sodium salt per os. (S=0.072 mg.) No albuminuria.
4	0.0784	0.0376	0.0031	0.0357	0.555	
5	0.0210	0.0091	0.0052	0.0067	0.557	
6	0.0185	0.0055	0.0054	0.0076	0.612	

origin in the sulfur of the compound administered, but which is associated with the increased amount of protein catabolized. In determining the "extra sulfur" in such cases, the value for the normal sulfur excretion should be calculated from the N:S ratio of the preliminary days and the total nitrogen of the experimental day. Thus in the case of Rabbit P (Table III) in which the administration of a relatively large amount of thioglycollic acid resulted in a marked increase in the excretion of total nitrogen, the computation of the "extra sulfur" due to the thioglycollic acid, would be as follows: On the 3 preliminary days the ratio of nitrogen to sulfur was 16.4. Applying this ratio to the experimental day, an elimination of 1.085 gm. of nitrogen would correspond to 0.066 gm. of sulfur. This would give 0.1419 gm. of "extra sulfur" originating from the thio compound fed or a recovery of slightly more than 100 per cent. The "extra" sulfate sulfur (inorganic and conjugated) calculated similarly becomes 0.074 gm. or an oxidation of about 52 per cent of the sulfur recovered in the urine. When one considers that this calculation is only an approximation, the agreement between the amounts of sulfur fed and recovered may be regarded as satisfactory.

Thiodiglycollic Acid, S (CH_2COOH)₂.—Tables VI to VIII record the results of feeding and injection experiments with thiodiglycollic acid. This compound produced no toxic symptoms whatever in the dose administered. The total nitrogen output did not vary significantly. Practically all the sulfur fed as thiodiglycollic acid was eliminated as organic sulfur. In five experiments (not all recorded in the tables) in which the acid was administered *per os*, 86, 82, 86, 79, and 83 per cent, respectively, of the sulfur was eliminated as "extra" sulfur in the first 2 days. In three experiments in which it was injected subcutaneously, 93, 100, and 68 per cent was eliminated as "extra" sulfur. No increase in sulfate sulfur excretion could be observed and no evidence of oxidation of the sulfur fed.

The reason for the difference in toxicity between thiolactic and thioglycollic acid is not clear. Both are oxidized to approximately the same extent despite the toxicity of the thioglycollic acid. Lusk (15) has suggested an oxidative desulfurization of cystine similar to oxidative deamination in order to explain the fact that all 3 carbon atoms of cystine are concerned with the

TABLE VI.

Rabbit I. Male. Weight 2.3 kilos. Daily diet: 150 cc. of milk, 10 gm. of sucrose, and 10 gm. of hay.

Day.	Total sulfur.	Inorganic sulfate sulfur.	Conjugated sulfate sulfur.	Organic sulfur	Total nitrogen.	Remarks.
	gm.	gm.	gm.	gm.	gm.	
1	0.0185	0.0055	0.0054	0.0076	0.612	
2	0.0203	0.0085	0.0044	0.0074	0.580	
3	0.0227	0.0055	0.0096	0.0076	0.548	{ 0.735 gm. thiodiglycollic acid as sodium salt per os. (S=0.157 gm.)
4	0.1590	0.0102	0.0072	0.1406	0.538	
5	0.0247	0.0081	0.0017	0.0149	0.508	
6	0.0233	0.0151	0.0019	0.0063	0.589	

TABLE VII.

Rabbit L. Male. Weight 2.3 kilos. Daily diet: 150 cc. of milk, 10 gm. of sucrose, and 10 gm. of hay.

Day.	Total sulfur.	Inorganic sulfate sulfur.	Conjugated sulfate sulfur.	Organic sulfur.	Total nitrogen.	Remarks.
	gm.	gm.	gm.	gm.	gm.	
1	0.0232	0.0095	0.0044	0.0093	0.515	
2	0.0221	0.0081	0.0037	0.0103	0.550	{ 0.276 gm. thiodiglycollic acid as sodium salt per os. (S=0.059 gm.)
3	0.0234	0.0093	0.0031	0.0110	0.493	
4	0.0695	0.0093	0.0040	0.0562	0.485	
5	0.0240	0.0060	0.0050	0.0130	0.480	
6	0.0221	0.0060	0.0055	0.0106	0.558	{ 0.251 gm. thiodiglycollic acid as sodium salt by subcutaneous injection. (S=0.054 gm.)
7	0.0235	0.0140	0.0039	0.0056	0.550	
8	0.0768	0.0150	0.0045	0.0573	0.450	
9	0.0299	0.0095	0.0064	0.0140		
10	0.0302	0.0182	0.0035	0.0085	0.502	

TABLE VIII.

Rabbit AA. Male. Weight 2.1 kilos. Daily diet: 150 cc. of milk, 10 gm. of sucrose, and 10 gm. of hay.

Day.	Total sulfur.	Inorganic sulfate sulfur.	Conjugated sulfate sulfur.	Organic sulfur.	Total nitrogen.	Remarks.
	gm.	gm.	gm.	gm.	gm.	
1	0.0286	0.0073	0.0095	0.0118	0.515	{ 0.284 gm. thiodiglycollic acid as sodium salt by subcutaneous injection. (S=0.061 gm.)
2	0.0276	0.0069	0.0104	0.0103	0.573	
3	0.0689	0.0028	0.0073	0.0588	0.408	
4	0.0203	0.0030	0.0066	0.0107	0.450	

formation of "extra glucose" in the phlorhizinized animal. If such a reaction occurred in the case of the two thio acids under discussion, hydrogen sulfide and either lactic or glycollic acid would be formed. Of these products hydrogen sulfide is rapidly oxidized in amounts far above the lethal dose (16) and lactic and glycollic (17) acids are without toxic effects. The failure of the sulfur of thiodiglycollic acid³ to be oxidized is in harmony with the theory that only in compounds containing the mercapto group or in compounds which can yield mercapto groups on reduction or hydrolysis is organic sulfur readily oxidized.

SUMMARY.

1. Thiolactic acid (as the sodium salt) when administered to rabbits either subcutaneously or *per os* was readily oxidized, yielding about 50 per cent of the sulfur eliminated in the urine as sulfate sulfur. In the quantities fed, it was entirely non-toxic.

2. The sulfur of thioglycollic acid similarly administered was oxidized only a little less readily than thiolactic acid. It was, however, toxic.

3. No oxidation of the sulfur of thiodiglycollic acid was observed after either oral or subcutaneous administration. No toxic action was noted.

4. It seems probable that of the different types of organic sulfur compounds, only those containing the mercapto group or those which can readily be transformed in the organism into compounds containing this group, are oxidized with ease in the animal organism.

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THE METABOLISM OF SULFUR.

VIII. THE BEHAVIOR OF THIOPHENOL AND THIOCRESOL IN THE ANIMAL ORGANISM.*

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(Received for publication, February 11, 1924.)

In the preceding paper (1) it has been shown that the sulfur of aliphatic mercapto groups, as in thiolactic and thioglycollic acids, is readily oxidized in the organism of the rabbit, while thiodiglycollic acid, in which sulfur is present as a sulfide, was not oxidized. It was considered probable that in sulfur compounds related to cystine, only the sulfur present as mercapto sulfur or sulfur which could readily be converted to mercapto sulfur in the organism was oxidized to sulfates to any marked degree.

In the present study, we have concerned ourselves with the behavior of mercapto groups attached to the benzene ring as in thiophenol, $C_6H_5 \cdot SH$, and *p*-thiocresol, $CH_3 \cdot C_6H_4 \cdot SH$. Despite the fact that these compounds are rather more toxic than are the aliphatic mercapto derivatives previously discussed and are not absorbed readily, we have obtained evidence which demonstrates that in the organism of the rabbit at least, *no appreciable oxidation of the sulfur of mercapto groups attached directly to the benzene ring occurred.*

EXPERIMENTAL.

The experimental animals, rabbits, and the conduct of the experiments were the same as in our experiments with aliphatic mercapto derivatives (1). The thiophenol and thiocresol were prepared for this work by the organic division of this university.

* An abstract of a thesis submitted by R. M. Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Graduate School of the University of Illinois.

The thiophenol was redistilled and analysis of its sulfur content proved satisfactory. The purity of the *p*-thiocresol was established by its melting point and sulfur determination. Neither of these substances is readily soluble in water. In the earlier experiments, the thiocresol was emulsified by shaking with water to which a few drops of 0.1 N sodium hydroxide and a very little vaseline had been added. This resulted in an emulsion sufficiently permanent to permit administration to animals. Later both the thiocresol and thiophenol were dissolved in olive oil and administered in this form.

TABLE I.

Rabbit G. Male. Weight 1.9 kilos. Daily diet: 150 cc. of milk, 10 gm. of sucrose, and 5 gm. of hay.

Day.	Total sulfur.	Inorganic sulfate sulfur.	Conjugated sulfate sulfur.	Organic sulfur.	Total nitrogen	Remarks.
	gm.	gm.	gm.	gm.	gm.	
1	0.0272	0.0150	0.0077	0.0045	0.538	
2	0.0282	0.0124	0.0092	0.0066	0.543	
3	0.0218	0.0112	0.0073	0.0033	0.537	
4	0.0190	0.0071	0.0082	0.0037	0.534	
5	0.0310	0.0056	0.0109	0.0145	0.629	{ 0.235 gm. thiophenol <i>per os</i> . (S = 0.068 gm.) Albumin negative. Hemoglobin negative. Pigment negative.
6	0.0243	0.0029	0.0128	0.0086	0.503	
7	0.0155	0.0015	0.0087	0.0053	0.593	

Thiophenol. Thiophenol proved to be toxic when administered to rabbits either orally or subcutaneously. As far as it is possible to judge from the elimination of "extra" sulfur in the urine, absorption did not occur readily either from the intestine or from the tissue into which injection was made. The maximum recovery of "extra" sulfur was noted with Rabbit Y (Table II) in which on the day following the subcutaneous injection of a small amount of thiophenol, approximately 20 per cent of the sulfur injected was eliminated as "extra" organic sulfur in the urine. In other experiments (Tables I to III), oral administration resulted in slight increases in the urinary organic sulfur, increases clearly above the normal fluctuations for this component. No increase in the sul-

TABLE II.

Rabbit Y. Male. Weight 2.8 kilos. Daily diet: 150 cc. of milk, 10 gm. of sucrose, and 10 gm. of hay.

Day.	Total sulfur.	Inorganic sulfate sulfur.	Conjugated sulfate sulfur.	Organic sulfur.	Total nitrogen.	Remarks.
	gm.	gm.	gm.	gm.	gm.	
1	0.0489	0.0251	0.0075	0.0163	0.960	
2	0.0529	0.0265	0.0085	0.0179	0.965	
3	0.0553	0.0294	0.0084	0.0175	0.880	
4	0.0593	0.0213	0.0097	0.0283	0.950	{ 0.180 gm. thiophenol <i>per os</i> . (S = 0.052 gm.) Albumin negative. Hemoglobin negative.
5	0.0437	0.0119	0.0116	0.0202	0.818	
6	0.0409	0.0170	0.0084	0.0155	0.800	
7	0.0563	0.0177	0.0094	0.0292	0.870	{ 0.187 gm. thiophenol subcutaneously. (S = 0.054 gm.) Albumin negative. Hemoglobin negative. Pigment negative.
8	0.0383	0.0113	0.0066	0.0204	0.855	

TABLE III.

Rabbit U. Male. Weight 2.9 kilos. Daily diet: 150 cc. of milk, 10 gm. of sucrose, and 10 gm. of hay.

Day.	Total sulfur.	Inorganic sulfate sulfur.	Conjugated sulfate sulfur.	Organic sulfur.	Total nitrogen.	Remarks.
	gm.	gm.	gm.	gm.	gm.	
1	0.0628	0.0375	0.0078	0.0175	1.048	
2	0.0646	0.0390	0.0070	0.0186	1.110	
3	0.0635	0.0354	0.0095	0.0186	1.035	
4	0.0596	0.0284	0.0101	0.0211	0.988	{ 0.117 gm. thiophenol <i>per os</i> . (S = 0.034 gm.) Albumin negative. Pigment negative.
5	0.0687	0.0415	0.0067	0.0205	1.160	
6	0.0667	0.0386	0.0092	0.0189	1.090	
7	0.0798	0.0468	0.0140	0.0190	1.272	{ 0.130 gm. thiophenol subcutaneously. (S = 0.038 gm.) Albumin negative. Pigment negative.
8	0.0667	0.0203	0.0132	0.0332	1.000	
9	0.0641	0.0212	0.0115	0.0314	1.300	

fate sulfur of the urine was noted with the possible exception of one experiment (Table III) in which a small quantity of thiophenol was injected. A previous oral administration of thiophenol to this animal had failed to increase the elimination of any of the various forms of urinary sulfur determined. The increase in the sulfate sulfur was very slight and was probably within the normal range, specially when it is noted that the total nitrogen was also increased. The increased sulfate sulfur which might be expected to be associated with this increase in nitrogen would account for practically all the increase in sulfate sulfur observed. No albuminuria or hemoglobinuria was observed even in those experiments in which lethal doses of thiophenol were administered.

TABLE IV.

Rabbit A. Male. Weight 1.9 kilos. Daily diet: 150 cc. of milk, 10 gm. of sucrose, and 5 gm. of hay.

Day.	Total sulfur.	Inorganic sulfate sulfur.	Conjugated sulfate sulfur.	Organic sulfur.	Total nitrogen.	Remarks.
	gm.	gm.	gm.	gm.	gm.	
1	0.0328	0.0233	0.0023	0.0072	0.484	
2	0.0328	0.0233	0.0023	0.0072	0.484	
3	0.0305	0.0234	0.0015	0.0056	0.592	
4	0.0450	0.0219	0.0045	0.0186	0.573	{ 0.27 gm. thiocresol <i>per os</i> . (S = 0.070 gm.) Albumin negative. Hemoglobin negative.
5	0.0306	0.0165	0.0029	0.0112	0.488	
6	0.0220	0.0126	0.0006	0.0088	0.507	

Thiocresol.—In Tables IV to VI are recorded the results of typical experiments in which *p*-thiocresol was fed or injected. Thiocresol produced marked toxic effects, accompanied by a severe albuminuria in some cases. Spectroscopic examination of the urine in a few experiments revealed the presence of oxyhemoglobin.¹ Although there was a marked individual variation in the reaction to thiocresol, in most cases in which albuminuria resulted,

¹ In the tables a few typical experiments only are reported. In all cases, these results were checked with other animals. In order to condense the data these additional experiments are omitted. They are, however, recorded in the original thesis on file in the Library of the University of Illinois.

a dark red pigment (not a blood pigment derivative as shown by spectroscopic examination) appeared in the urine. The presence of this unknown pigment in the urine corresponded roughly to the toxic effect of the thiocresol, but seemed to bear no relation to the

TABLE V.

Rabbit M. Male. Weight 2.3 kilos. Daily diet: 150 cc. of milk and 10 gm. of sucrose.

Day.	Total sulfur.	Inorganic sulfate sulfur.	Conjugated sulfate sulfur.	Organic sulfur.	Total nitrogen.	Remarks.
	gm.	gm.	gm.	gm.	gm.	
1	0.0288	0.0126	0.0052	0.0110	0.773	
2	0.0284	0.0159	0.0043	0.0082	0.822	
3	0.0198	0.0084	0.0044	0.0070	0.748	
4	0.0286	0.0136	0.0056	0.0094	0.777	
5	0.0489	0.0132	0.0052	0.0305	0.802	0.3 gm. thiocresol per os. (S = 0.078 gm.) Strong albumin. Strong pigment. No food taken. " " " Rabbit very weak.
6	0.0190	0.0037	0.0092	0.0061	0.407	
7	0.0147	0.0036	0.0067	0.0044	0.287	

TABLE VI.

Rabbit E. Male. Weight 2.1 kilos. Daily diet: 150 cc. of milk, 10 gm. of sucrose, and 5 gm. of hay.

Day.	Total sulfur.	Inorganic sulfate sulfur.	Conjugated sulfate sulfur.	Organic sulfur.	Total nitrogen.	Remarks.
	gm.	gm.	gm.	gm.	gm.	
1	0.0253	0.0125	0.0049	0.0079	0.583	
2	0.0244	0.0114	0.0051	0.0079	0.539	
3	0.0327	0.0205	0.0057	0.0065	0.630	
4	0.0253	0.0114	0.0038	0.0101	0.653	0.355 gm. thiocresol subcutaneously. (S = 0.092 gm.) Albumin negative. Hemoglobin negative. Slight pigment.
5	0.0354	0.0102	0.0055	0.0197	0.780	
6	0.0253	0.0089	0.0062	0.0102	0.786	

hemoglobin since it was present when no test for hemoglobin could be obtained. Attempts to isolate this pigment will be discussed later. There was no evidence of any oxidation of the sulfur of the thiocresol in any of the experiments. The "extra" sulfur recovered

in the organic sulfur fraction in no case corresponded to more than a very small fraction of that administered, but was clearly above the normal variation in every instance.

This failure to recover more than a small amount of the sulfur of the compounds from the urine was apparently due to failure of absorption. After oral administration, the ether extract of the feces gave the orange precipitate with lead acetate which is characteristic of thiocresol. After subcutaneous injection, a hard lump was frequently formed at the site of injection. At autopsy the thiocresol appeared to have crystallized in the tissues at the site of the injection and its presence in ether extracts of such tissues was determined by the lead acetate reaction.

An attempt was made to isolate the urinary pigment which was present after administration of thiocresol. The pigment could be extracted from the urine by ether or chloroform. On evaporation of these extracts, a bright, purple-red material remained. When the urines which contained this pigment were examined spectroscopically no absorption bands could be observed. It was, therefore, not a hemoglobin derivative. The extracted pigment was tested for sulfur by the usual sodium fusion method with negative results. Inasmuch as the amounts obtainable from the urine were so small, no further study of the pigment was made.

It seemed possible that if the mercapto groups of the compounds studied were not oxidized or split from the ring, the organism might protect itself against the toxic thiophenol nucleus by conjugation with sulfuric acid, as is the case with phenol (2). This would give rise to an increase in the conjugated sulfate sulfur of the urine and would result in the presence of conjugated thio-sulfates of the type $C_6H_5 \cdot S \cdot SO_2 \cdot OH$. No evidence that the organism availed itself of any such mode of detoxication could be obtained.

SUMMARY.

1. After the administration of *p*-thiocresol and thiophenol to rabbits, there was no evidence that the sulfur of the mercapto groups was oxidized to sulfate sulfur. Despite the insolubility of these thiophenols, evidence of partial absorption was obtained from an increase in the organic sulfur fraction of the urine. This failure of oxidation of the sulfur of mercapto groups attached to the

benzene ring was in marked contrast to the behavior of mercapto groups in aliphatic compounds in which oxidation of the sulfur readily occurred (1).

2. Thiophenol and thiocresol are toxic. After the administration of thiocresol, a red pigment containing no sulfur and probably not a hemoglobin derivative (no absorption spectrum) appeared in the urine.

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AMINO ACIDS IN NUTRITION.

VIII. PROLINE IS INDISPENSABLE FOR GROWTH.*

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(Received for publication, February 6, 1924.)

For his plan to study the rôle of proline in nutrition the author originally chose three proteins; namely, arachin, lactalbumin, and edestin. Lactalbumin had to be abandoned for proline studies, because cystine was found to be the primary growth-limiting factor in that protein (1). Arachin (a globulin and the main protein from the peanut) was a physiological puzzle. It was primarily selected on account of its low proline content. From the standpoint of chemical composition arachin should be a fairly good protein, but even after being fortified with cystine and proline it served for nothing better than maintenance; neither did the leucine fraction (composed of alanine, leucine, and valine) nor tryptophane improve its biological value (2). The theory was then proposed that arachin is stereochemically unbalanced; in other words, that the trouble with that protein is not so much with the composition of the amino acids as with their physical arrangement, so that on digestion the cleavage products escape absorption and are mainly converted into urea. Recently, Jones and Waterman produced chemical evidence in support of that hypothesis (3).

In order successfully to employ edestin (the globulin from hempseed) for the study of the rôle of the pyrrolidine nucleus in nutrition, it was necessary first to find out other possible amino acid deficiencies in that protein, so that proline should represent the

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only variable constituent in the synthetic rations. By the introduction of a modified method of procedure (4), cystine and lysine were found to be at least two amino acids deficient in edestin (5), Osborne and Mendel having previously demonstrated lysine to be at least one of the growth-limiting factors in that protein (6). Since the remarkable response to cystine was obtained only in the presence of gelatin, the next problem that suggested itself was, by virtue of which amino acids does gelatin owe its supplementary value to edestin? Gelatin, although it is deficient in cystine, tyrosine, and tryptophane, is quite abundant in proline and arginine; therefore, experiments were initiated with the idea of employing edestin as the basal protein (at different planes of intake) fortified with cystine and lysine, with the expectation of noting the difference in growth produced by the further addition of proline, and if necessary, by the addition of proline in the presence of arginine.

The results of the experiments are summarized in Charts I to VIII and Tables I to VI, which are analyzed in the following discussion.

DISCUSSION.

Charts I and II and Tables I and II indicate that no particular advantage was derived by the introduction of proline when edestin was fed at a 9 per cent plane of protein intake. The small increase in gain in weight per gram of protein intake of Lot 298 over Lot 297 may be attributed to experimental error. The response to proline, however, became very pronounced when the level of protein intake was reduced to 6 per cent. Chart III clearly shows how two animals completely failed on a 6 per cent edestin-cystine-lysine diet, while Chart IV shows the enormous growth manifested by two animals on a 6 per cent edestin-cystine-lysine-proline ration. After 12 weeks experimentation, however, it was not very apparent whether two animals on the proline-containing ration were making any better growth than on the control diet in which proline was absent. In a previous communication (5) the author stated his experience with respect to the behavior of individual animals in responding to specific amino acid additions. The statement was made that individuals differ in their capacity for conserving the building blocks of the

protein molecule. It was anticipated, then, that the animals that had not responded to proline probably needed more arginine. The abnormal behavior of individuals with regard to the utiliza-

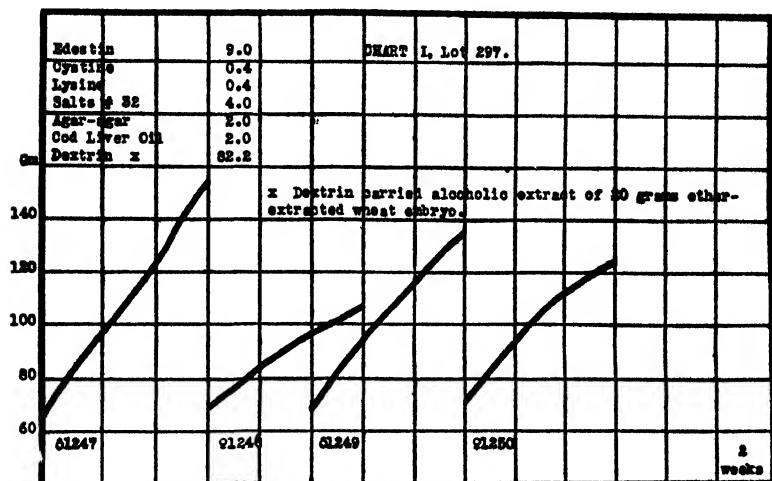


CHART I.

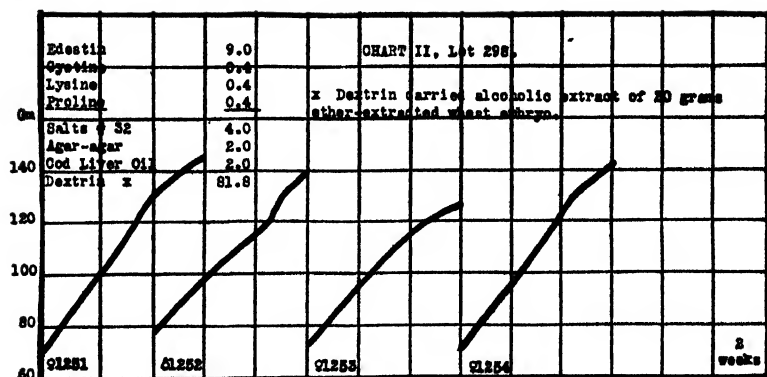


CHART II.

tion of arginine may be related to creatine and creatinine metabolism. Gross and Steenbock (7) have shown how the introduction of arginine, which contains the guanidine nucleus, results in the increase of creatine excretion in the pig; and the large

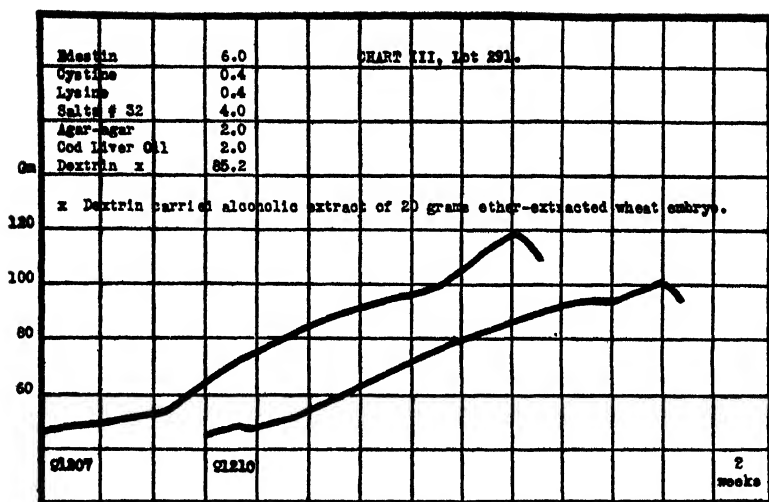


CHART III.

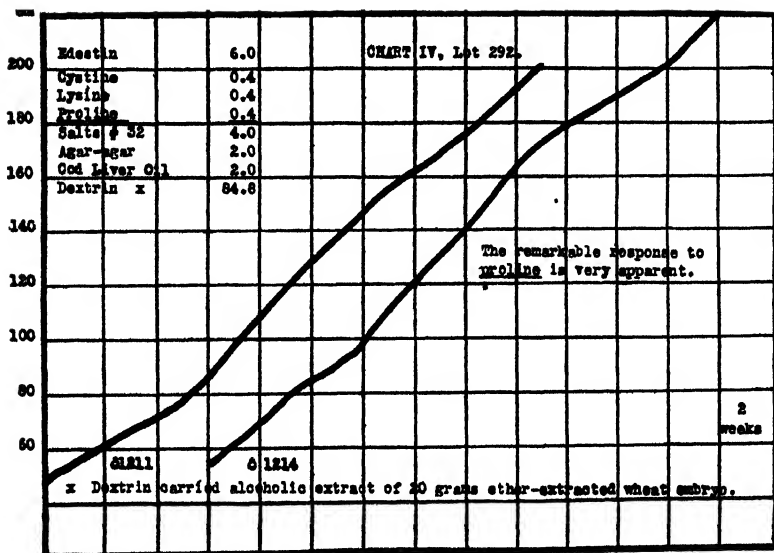


CHART IV.

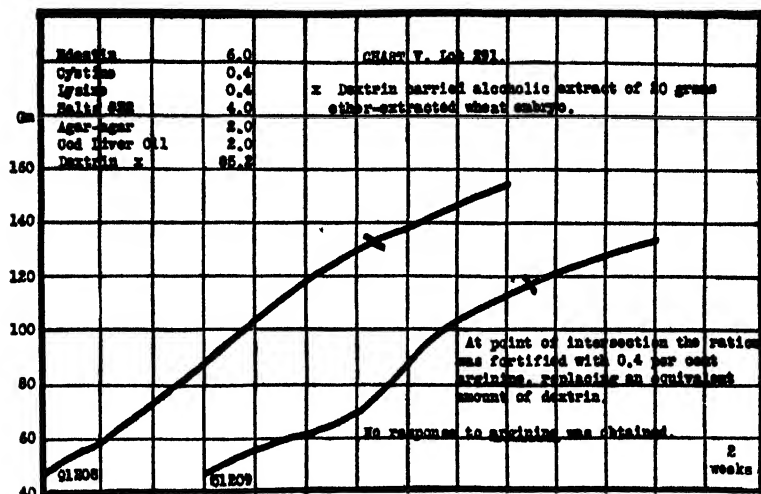


CHART V.

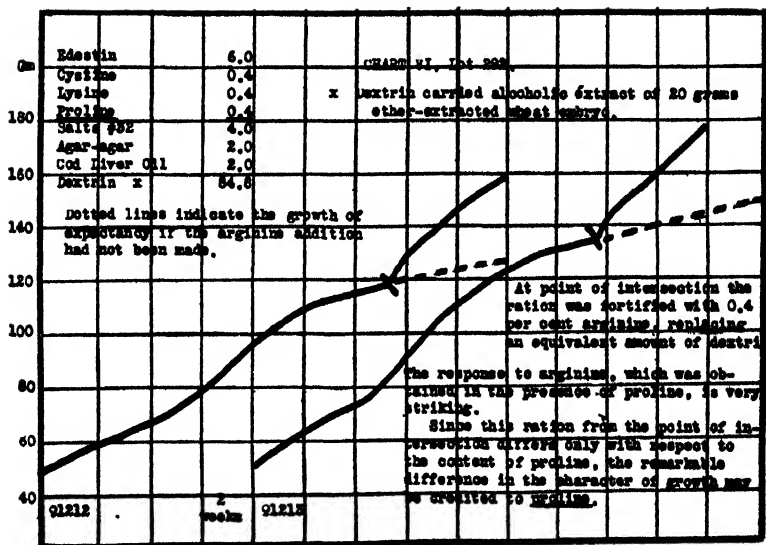


CHART VI.

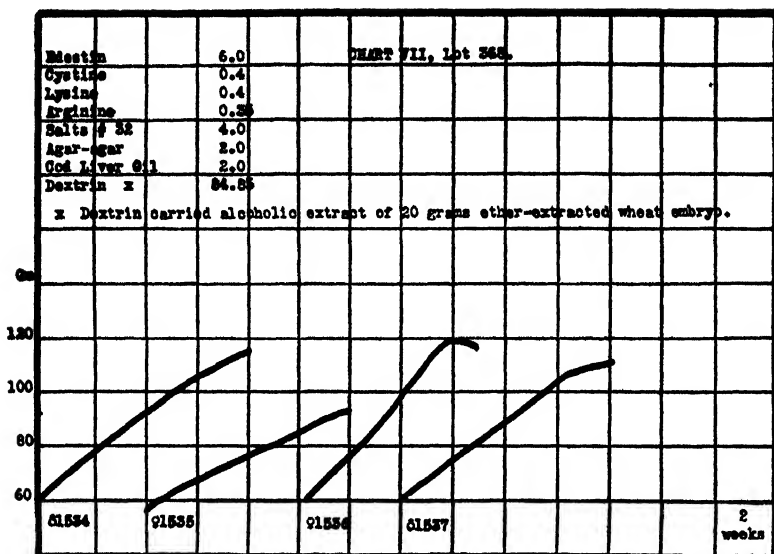


CHART VII.

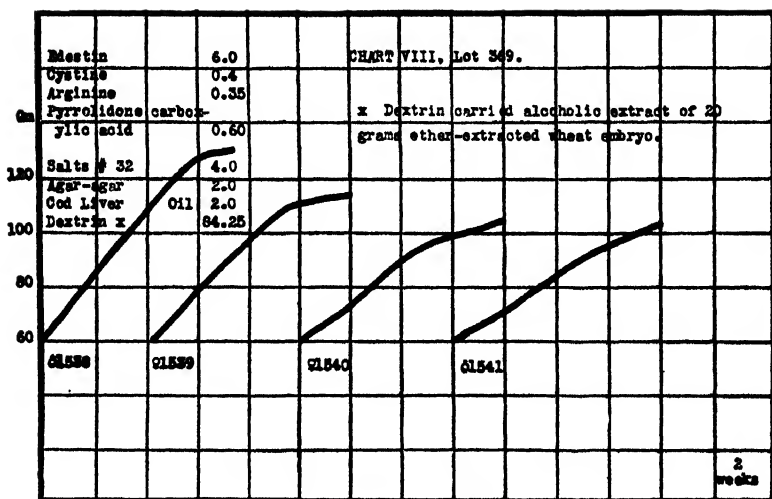


CHART VIII.

differences in the amount of creatine output among individual animals may very well be related to the peculiar influence of the enzyme arginase in splitting the arginine molecule, and liberating the guanidine ring, which, after methylation, may be converted into creatine. If the greater part of arginine contained in a pro-

TABLE I.
Showing Gains in Weight in Grams.

Protein.	^u Lot No.	Rat No.
<i>per cent</i>		
Edestin..... 9 0	297	1247
Cystine..... 0 4		1248
Lysine..... 0 4		1249
		1250
Edestin..... 9 0	298	1251
Cystine..... 0 4		1252
Lysine..... 0 4		1253
Proline..... 0 4		1254

TABLE II.
Showing Gain in Grams per Gram of Protein Intake.

Protein	Lot No.	Gain in weight.	Amount of protein consumed.	Gain per gram of protein intake.
<i>per cent</i>		<i>gm.</i>	<i>gm.</i>	<i>gm.</i>
Edestin..... 9 0	297	228	221 4	1.03
Cystine... 0 4				
Lysine..... 0 4				
Edestin..... 9 0	298	236	217 35	1 08
Cystine..... 0 4				
Lysine..... 0 4				
Proline..... 0.4				

tein is converted by certain individual animals into creatine, then a greater amount of arginine of a protein will be required by those individuals for the construction of body tissue. This speculation was advanced in spite of the fact that arginine is rather abundant in edestin. Jones and Waterman (3) have

TABLE III.
Showing Gains in Weight in Grams.

Protein.	Lot No.	Rat No.	Sex.	Weight of animals.											
				Initial.	2 wks.	4 wks.	6 wks.	8 wks.	10 wks.	12 wks.	14 wks.	16 wks.	18 wks.	19 wks.	
<i>per cent</i>				gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	
Edestin.... 6.0	291	1207	♀	47	53	56	63	77	82	90	96	104	120	112	
Cystine.... 0.4		1210	♀	48	50	57	62	70	77	85	90	96	103	97	
Lysine..... 0.4		1208	♀	52	58	70	86	102	119	131*	141	152	158		
		1209	♂	49	57	61	66	88	102	115*	127	134	138		
Edestin.... 6.0	292	1211	♂	47	57	70	87	110	127	148	159	175	193	201	
Cystine.... 0.4		1214	♂	55	71	76	98	119	140	163	176	187	204	220	
Lysine..... 0.4		1212	♀	49	58	64	76	94	102	105*	140	152	160		
Proline.... 0.4		1213	♀	48	61	70	92	112	121	134*	149	165	175		

* At this point animals Nos. 1208, 1209, 1212, and 1213 received 0.4 per cent arginine in the ration. The response to arginine, in the presence of proline, is quite apparent.

TABLE IV.
Gain in Weight per Gram of Protein Intake after the 12th Week Weighing When the Animals in Lots 291 and 292 Were Separated in Individual Cages and Records of Individual Food Consumption Taken.

Protein. <i>per cent</i>	Lot No.	Rat No.	Sex.	Gain in weight.	Amount of protein consumed.	Gain per gram of protein intake.
				gm.	gm.	gm.
Edestin..... 6.0	291	1207	♀	17.0	23.46	0.73
Cystine..... 0.4		1210	♀	11.0	21.06	0.52
Lysine..... 0.4						
Edestin..... 6.0	292	1211	♂	50.0	36.0	1.46
Cystine..... 0.4		1214	♂	52.0	37.5	1.38
Lysine..... 0.4						
Proline..... 0.4						
Edestin..... 6.0	291	1208	♀	24.0	24.60	0.98
Cystine..... 0.4		1209	♂	20.0	22.32	0.89
Lysine..... 0.4						
Arginine..... 0.4						
Edestin..... 6.0	292	1212	♀	43.0	27.12	1.58
Cystine..... 0.4		1213	♀	40.0	28.20	1.42
Lysine..... 0.4						
Arginine..... 0.4						
Proline..... 0.4						

demonstrated that the hexone bases of arachin are resistant towards enzyme action and a similar situation with regard to the

TABLE V.
Showing Gains in Weight in Grams.

Protein.	Lot No.	Rat No.	Sex.	Weight of animals.					
				Initial.	2 wks.	4 wks.	6 wks.	7 wks.	8 wks.
per cent				gm.	gm.	gm.	gm.	gm.	gm.
Edestin..... 6 0	368	1534	♂	61	77	90	106		112
Cystine..... 0.4		1535	♀	57	69	77	85		94
Lysine..... 0.4		1536	♀	61	78	102	119	117	
Arginine..... 0.35		1537	♂	62	76	92	104		111
Edestin..... 6 0	369	1538	♂	61	82	107	128	129	
Cystine..... 0.4		1539	♀	62	80	99	111		117
Lysine..... 0.4		1540	♀	60	71	84	96		106
Arginine..... 0.35		1541	♂	60	71	85	98		103
Pyrrolidone carboxylic acid..... 0 6									

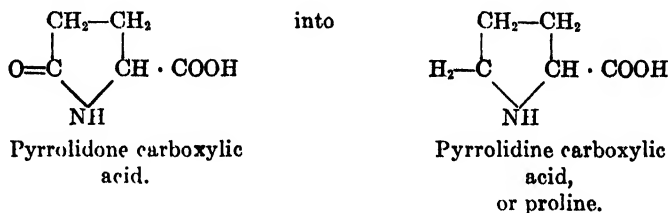
TABLE VI.
Showing Gain in Grams per Gram of Protein Intake.

Protein.	Lot No.	Rat No.	Sex.	Gain in weight.	Amount of protein consumed.	Gain per gram of protein intake.
per cent				gm.	gm.	gm.
Edestin..... 6 0	368	1535	♀	37.0	23.16	1.17
Cystine..... 0.4		1534	♂	52.0	27.96	1.86
Lysine..... 0.4		1536	♀	56.0	28.20	1.98
Arginine..... 0.35		1537	♂	49.0	26.82	1.82
Edestin..... 6 0	369	1538	♂	68.0	30.00	2.26
Cystine..... 0.4		1539	♀	55.0	30.00	1.83
Lysine..... 0.4		1540	♀	56.0	25.50	1.84
Arginine..... 0.35		1541	♂	43.0	25.20	1.70
Pyrrolidone carboxylic acid. 0.4						

digestibility of arginine in edestin was anticipated; therefore, the following modifications in the proline experiments were made.

At points of intersection in Charts V and VI the rations of Lots 291 and 292 were fortified with 0.4 per cent arginine, replacing an equivalent amount of dextrin. Since, after the incorporation of arginine in the rations, the animals were confined in individual cages, and individual records were kept of food consumption, data on the gain per gram of protein intake for each animal are available, and Table IV shows the striking response to the pyrrolidine nucleus which was secured in the presence of arginine. Responses to proline were secured on 6 per cent edestin levels, in the presence of cystine and lysine in some animals, and in the presence of cystine, lysine, and arginine in others. *Proline is, then, considered by the author as an essential amino acid for growth.*

Charts VII and VIII and Tables V and VI show that the animal organism has not the capacity of transforming



The tables and charts show one abnormal animal on Ration 368, which may be disregarded in the interpretation of the data.

SUMMARY.

1. *Proline is an essential amino acid for growth.*
2. The animal organism has not the capacity of transforming pyrrolidone into pyrrolidine carboxylic acid.

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DIET IN RELATION TO REPRODUCTION AND REARING OF YOUNG.*

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The object of this work is twofold: first, to determine if a diet which contains milk or milk products as the sole source of proteins and vitamins is detrimental to the normal growth impetus of the animal and especially the reproductive function; and second, to determine the value of various levels of wheat embryo as the sole source of vitamin B on growth, reproduction, and the rearing of the young.

Recent experiments by Evans and Bishop (1) show that an unknown dietary factor, different from the well known vitamins, is necessary for reproduction. The diet used by these investigators consisted of food substances which have been recognized by workers in nutrition as supplying all the necessary constituents for growth and reproduction. Evans and Bishop (1) make the statement that animals fed their synthetic diets containing yeast as the sole source of vitamin B are for the most part sterile in the first generation and wholly so in the second generation. They believe that an unknown substance, X, is necessary for successful reproduction. Kennedy and Palmer (2) likewise employed purified diets, supposedly sufficient for the needs of the animal body, but few of the animals reproduced. The results obtained by these investigators, as far as reproduction is concerned, were analogous to those of Evans and Bishop (1). One of the conclusions reached by Kennedy and Palmer was that yeast is not a very rich source of vitamin B. Heller (3) of this laboratory demonstrated subsequently that yeast contains an abundance of this particular vitamin. In one of a series of articles published recently from this laboratory by Nelson, Heller, and Fulmer (4) it was shown that it is not necessary to assume the existence of an unknown dietary factor for reproduction. Rats receiving a synthetic diet in which yeast furnished the only source of vitamin B reproduced on

* This paper forms part of a thesis to be submitted to the Graduate Faculty of the Iowa State College in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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levels of yeast as low as 1.5 per cent. The surprising observation was made, however, that not all the young were reared on the various levels of yeast employed. Third generation animals were weaned when the diet contained 5 per cent of yeast as the sole source of vitamin B, although by no means were all the young reared on this level of vitamin B intake. The young which were not reared on the various levels of yeast died in an emaciated condition in a period of time varying from 1 to about 4 weeks. Although growth in the first generation was normal or better, the young did not behave in a normal manner even though they were normal in weight at birth and, as far as could be learned, perfectly healthy. The impression prevailed that something was wrong either in the chemical composition of the milk or in the physiology of milk secretion.

Recent work by Hartwell (5) on the relation of diet to mammary secretion indicates that excessive protein in the diet of the mother during the lactation period is detrimental to the well-being of the suckling. Mattill and Stone (6) have published, recently, some work bearing on this general problem. They state that on diets in which all the protein and vitamins were given in the form of dry milk rats grew at a normal rate up to 75 days of age and that subsequently the rate fell below normal, the decline being more marked in females than in males. At 175 days of age the females were 14 per cent underweight and the males were 5 per cent below the average weight. Reproduction was not successful on any of the diets employed, and they suggest that the reproductive failure on milk rations might be explained by the unknown dietary factor, X, which Evans and Bishop (1) maintain is necessary for normal reproduction.

Sugiura and Benedict (7) showed that one of their diets, which was sufficient for normal growth, failed during the period of lactation. When 10 cc. of whole milk were added to the diet it became adequate for milk production, a fact which led the above investigators to suggest that milk contains a new accessory factor which is needed by the mother in order that suitable milk might be formed.

EXPERIMENTAL AND DISCUSSION.

Vigorous rats weighing from 50 to 60 gm. were employed in these experiments. Prior to being placed on the experimental diets, the animals received an adequate growing ration with whole milk as a supplement, which was sufficient for normal growth and reproduction. The animals were weighed every 7 days, and their general condition and behavior were noted several times daily. Curves of growth and reproduction records were compared with results obtained on a normal diet. The whole and skim milk powders were Merrell-Soule products. Whole milk powder contained 26.40 per cent of protein, 27.5 per cent of

fat, and 6.0 per cent of ash. Powdered skim milk had the following amounts of protein, fat, and ash; namely, 37.00, 1.30, and 8.00 per cent, respectively.

The experiments of Mattill and Stone (6) suggested that something was lacking in diets composed of dried milk, and for this reason some of the rations carried various supplements such as additional protein in the form of casein, inorganic constituents in the form of a complete salt mixture, or agar-agar as roughage. The casein was thoroughly washed with water acidified with acetic acid until free from vitamin B. McCollum's salt mixture, No. 185 (8), was used. It is well known that milk is deficient in iron, and so where necessary the diet carried 0.2 gm. of iron citrate per 100 gm. The results obtained are given in Table I, and the composition of the diets is shown in Table II. Columns 2, 3, 4, 5, 14, and 15 (Table I, horizontal columns) refer to the first generation animals which were 5 to 6 weeks of age when placed on the diets. Column 13 gives the number of third generation young born; the other figures refer to the second generation young. Comparatively few of the second generation were brought to maturity and allowed to reproduce because of the large number of animals employed in these experiments. The number of females used for this purpose is given in Column 12. The object in carrying the animals through the second generation was to determine whether or not the theory of Evans and Bishop (1) applied, for they had stated that on their diets rats were for the most part sterile in the first generation and wholly so in the second generation. In order to save space growth curves have been omitted, but the character of the curve of growth is recorded in Table II. The symbols ++ show that the animals grew better than the normal rate, + denotes normal growth, and - refers to growth below the normal rate.

The data in Table I show that rats are not sterile, and they grow at the normal rate when the diet contains whole milk powder as the sole source of protein and vitamins. Second and third generation animals were reared when the diets contained from 50 to 99 per cent of whole milk powder. It is also evident from the table that a large number of the young died. Some were born dead, but the majority of the young which died succumbed in the first 48 hours of life. The difference between the figures showing

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the total number of young which died and the sum of the young born dead and those which died in 48 hours represents the young which died later. Nelson, Heller, and Fulmer (4) showed in a previous article that on a synthetic diet containing yeast as the sole source of vitamin B the young died in an emaciated condition

TABLE I.

Data Showing the Results Obtained on Whole and Skimmed Milk Powders.

Ration No.	No. of females.	No. of males	No. of females that died.	No. of males that died.	No. of litters.	Total No. of young born.	No of young that died.	No of young born dead	No of young that died in 48 hrs	No of young weaned.	No of 2nd generation females for reproduction.	No of 3rd generation.	Age of mothers at 1st litter.	On ration.	
													days	mos	
1	9	5	3	0	12	70	47	2	33	23	2	46	90	7	
2	5	3	0	1	13	97	9	1	8	88	4	17	80	6	
3	8	6	2	0	20	129	53	3	35	76	6	73	90	7	
4	9	5	0	0	30	235	33	2	30	202	7	138	90	7	
5	4	2	0	0	4	37	12	0	12	25			100	3	
6	4	2	0	0	7	58	19	6	13	39			100	5½	
7	7	3	2	1	10	52	17	0	17	35	5	54	107	7	
8	8	2	1	0	7	48	17	0	10	31	5	5	120	6	
9	4	3	1	1	3	15	11	6	5	4			122	5½	
10	4	3	1	2	5	22	10	3	7	12			115	5½	
11	7	3	0	1	6	46	7	2	2	39			95	6	
12	6	3	0	0	0	0	0	0	0	0	0	0		8	
13	7	2	0	1	16	97	21	1	20	76	4	24	95	8	
14	5	3	0	0	12	88	38	3	34	50			90	6	
15	7	3	1	0	0	0	0	0	0	0	0	0		6	
16	6	3	1	1	3	20	10	2	8	10			180	6	
17	5	3	1	0	1	1	1	0	0	0	0	0	90	3½	
18	5	3	1	1	6	32	25	0	25	7	5		150	7	

in from 1 to 4 weeks from the time of birth. Whatever the cause may be, the fact that the mortality of the second generation young is so high shows that these diets are not optimum even though the first generation may grow at the normal rate. The mortality records were repeated by the second and third generation animals and even accentuated because of the lowered vitality of succeed-

ing generations. The table shows also that the age of the mothers at the time of appearance of the first litter varied from 80 to 180 days. Numerous experiments conducted in this laboratory reveal the fact that many experimental diets, although sufficient for normal growth and reproduction, fail to provide adequately for

TABLE II.
Composition of Diets.

Ration No.	Whole milk powder.	Skimmed milk powder.	Casein.	Salt mixture.	Iron citrate.	Agar-agar.	Dextrin.	Starch.	Butter fat.	Lard.	Curve of growth.	Calories per gram of ration.
	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent		
1	70				0.2	4.0	25.8				++	4.70
2	60			2.4	0.2	4.0	33.4				++	4.48
3	60		6.0		0.2	4.0	29.8				++	4.58
4	60		6.0	2.4	0.2	4.0	27.4				++	4.46
5	60				0.2	4.0	35.8				++	4.58
6	60		6.0	4.2	0.2	4.0	25.6				++	4.40
7	*										++	
8	95				0.2	4.8					+	4.94
9	99.8				0.2						+	5.18
10	94				0.2	5.8					+	4.89
11	90		9.8		0.2						++	5.13
12	50			2.0				38.0		10.0	+	5.09
13	50			2.0		4.0	44.0				+	4.40
14	50		20.0	2.0				18.0		10.0	++	5.09
15		90			0.2	4.8			5.0		+	3.84
16		80			0.2	4.0			5.0	10.8	+	4.41
17		77			0.2	4.0			18.8		-	4.59
18		65		1.0	0.2	4.0	13.8		16.0		++	4.78

* Whole milk powder, liquid whole milk, and agar-agar, each *ad libitum*.

the rearing of the young. The mortality during the suckling period is high.

Comparatively little is known about the relation of diet to reproduction, rearing of the young, and normal milk secretion. Most of the work in nutrition has been concerned with growth, but the reproductive phase and the relation of diet to milk secre-

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tion are equally important. Data along this line should be applicable to studies in infant mortality and conditions involving abnormal functioning of the mammary gland so prevalent in the human being. The results obtained emphasize the necessity of nutrition experiments being placed on a quantitative basis to determine the levels of the various components of the diet for normal growth, reproduction, rearing of the young, and normal milk secretion. Fulmer, Nelson, and Sherwood (9) have demonstrated recently that the amounts of the individual constituents comprising the nourishment of the cell are of fundamental importance. These investigators showed that for yeast growth there is an optimum concentration for each individual constituent of the medium. Since the animal body is for the most part nothing more than a collection of cells, it is probable that within limits the same principle applies.

The table shows also that 155 litters consisting of 1,047 second generation young were born on the various powdered milk diets and 330 of the young died; 259 of the latter died 48 hours after birth. The fact that the young died before they were weaned does not mean that the animals used for these experiments were of low vitality, nor should it necessarily be implied that milk is not rich in some substances necessary for normal milk secretion. The experiments show that if optimum concentrations of the various constituents are secured, normal behavior will in all probability result.

Rats on Ration 1 received whole milk powder 70 per cent, the remainder of the diet consisting of dextrin, agar-agar, and iron citrate. The females gave birth to 70 young, 47 of which died before they were weaned. Notwithstanding the high mortality of the young, fourth generation animals have been reared on this diet. The fact that the young die indicates that the ration is not optimum, since this does not occur on a diet of wide selection. Three of the mothers of the first generation died giving birth to young. The young were normal in appearance. The third mother died without evidence of pregnancy. One mother had four litters of which only one was reared. Although one female had four litters, the general tendency on this amount of milk powder is for the mother to have fewer litters. Each litter contained the normal number of young.

Animals on diet No. 2, containing 60 per cent of whole milk powder supplemented with iron citrate, agar-agar, salt mixture, and dextrin, appeared in fine condition, grew better than the normal rate, and reproduced normally. The mortality on this diet was very low. The only difference between Diets 3 and 4 is in the salt mixture. Animals on Diet 3 received only iron citrate as the salt supplement, whereas the other group received in addition 2.4 per cent of Salt Mixture 185. Rats on Diet 4 produced more young and more frequently with a lower mortality than did the animals on Diet 3.

Diets 12, 13, and 15 are of particular interest. Diet 15 consisted of skimmed milk powder 90 per cent, Agar-agar 4.8 per cent, butter fat 5 per cent, and iron citrate 0.2 per cent. Although growth was at the normal rate, not a single one of the seven females had young. In the case of Diet 12 which contained whole milk powder 50 per cent, starch 38 per cent, lard 10 per cent, and salt mixture 2 per cent, growth was normal but no young were produced. This diet has the same composition as that recorded by Mattill and Stone (6). The poor results in this latter case are due to the high content of fat. Diet 13 contains the same amount of whole milk powder as No. 12, but a portion of the fat has been replaced by dextrin and agar-agar. A normal curve of growth resulted and the first litters were successfully weaned. Subsequent litters have been fewer in number, and the mortality has been higher. Not only did the animals fail to reproduce on 90 per cent of skimmed milk powder, but either reproduction or rearing of the young was not normal on 65, 77, and 80 per cent of skimmed milk powder. The latter quantities were supplemented with filtered butter fat, agar-agar, and iron citrate, and in the case of the diet containing 80 per cent of powder 10.8 per cent of lard was added. Not only was the mortality high, but the time of appearance of the first litters was much delayed. Diet 18 contains approximately the same composition as Diet 4 with which good results were obtained.

The tendency of workers in nutrition to add various constituents to the diet without consideration of the proportion of the constituents is not a sound practice. Evidence obtained in this laboratory shows that it is a matter of considerable importance that the ratio of fat to protein be within certain limits if optimum

results are expected. If the proportion of fat to protein is too high growth may be normal in the first generation, but the animals produce few or no young. Evans and Bishop (1) and Mattill and Stone (6) employed diets in which the ratio of fat to protein was too high for best results, and as a consequence few or no young were produced. It is possible that when the ratio of fat to protein is too high, the calorific value of the diet being also high, the animals consume a smaller amount of the diet with the result that the protein consumption is decreased to a point at which normal reproduction and even normal growth is impossible, due to a deficiency of certain amino acids at this level of protein intake. If this is the correct interpretation, then the proportion of fat that can be used successfully should vary with the quality of protein employed.

In a recent paper from this laboratory by Nelson, Heller, and Fulmer (4) it was shown that when 5 per cent of Salt Mixture 185 is employed in the diet along with 18 per cent of casein as the sole source of protein the animals have few or no young, even though the first generation grows at the normal rate. However, the amount of salt mixture the animal can tolerate is dependent on the nature and amount of the protein in the diet. In Experiment 2 (Table I) the total amount of salt mixture in the diet is 6.2 per cent and yet the animals reproduced normally and the mortality was low. The amount of protein in this diet is less than 16 per cent. On the other hand with 18 per cent of casein and 5 per cent of salt mixture in the diet no young are produced, although normal reproduction and rearing of the young take place when the salt mixture is reduced to 3.7 per cent. Growth and reproduction were normal on a diet containing 83.3 per cent of whole milk powder, 9 per cent of casein, 3.5 per cent of salt mixture, (185), 4 per cent of agar-agar, and 0.2 per cent of iron citrate. 5 females had 7 litters of 37 young, 29 of which were weaned. Third generation rats have been reared on this diet. This diet contained 31.0 per cent of protein, 22.90 per cent of fat, and 8.69 per cent of ash. The diet contained 4.70 calories per gm. The rats did well notwithstanding the high content of ash and fat, because of the high protein content of the diet. The animals reproduced and reared a considerable number of young even though the ash content of the diet was more than 60 per cent greater than the amount which

Nelson, Heller, and Fulmer (4) stated would cause failure during the period of reproduction when casein is used to the extent of 18 per cent.

The composition of Diet 14 is the same as that of Diet 12 with the exception of the casein. The calorific value of the two diets is the same. 50 of the 88 young born were successfully weaned. This diet contains 5.00 per cent of ash, 23.80 per cent of fat, 37.00 per cent of carbohydrate, and 33.20 per cent of protein. 4 males and 6 females on a diet of whole milk powder 85 per cent, iron citrate 0.2 per cent, agar-agar 4.0 per cent, and dextrin 10.8 per cent, had 5 litters, the young of which were all dead within 48 hours. This diet contained salts 5.30 per cent, fat 23.40 per cent, and protein 22.40 per cent. The diet contained 4.87 calories per gm. 4 males and 6 females received a diet consisting of whole milk powder 85 per cent, salt mixture 3.5 per cent, iron citrate 0.2 per cent, agar-agar 4 per cent, and dextrin 7.3 per cent and, although the animals are 8 months old, not a single one has been pregnant. This diet contained inorganic constituents to the extent of 8.8 per cent, fat 23.40 per cent, and protein 22.40 per cent.

Rats on Diet 7 grew at a rate above normal, and third generation young were reared. Animals on Diet 8 consisting of whole milk powder, agar-agar, and iron citrate produced normal young and a normal number to the litter. The period of reproduction was delayed. Animals on Diet 8 received a limited quantity sufficient only to give a normal curve of growth. Rats receiving a satisfactory powdered whole milk diet grow very rapidly, and it was believed that the failure in reproduction observed by other investigators might be the result of this rapid growth. The high mortality on Diet 9 is striking. The period of reproduction was delayed considerably, few litters were produced, and the number to the litter was small. The young which were reared appeared normal.

Hartwell (5) showed that young were not reared when the mothers were given high protein diets at the time of lactation. The young went into spasms and examination of the alimentary tracts showed a cessation of the flow of milk. It has been observed repeatedly in this laboratory also that diets high in protein and comparatively low in fat are detrimental to the rearing of the young. A considerable number of the young die without evidence

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of spasms as shown by Hartwell. They seem to die in an emaciated condition similar to that described by Nelson, Heller, and Fulmer (4). It was deemed advisable in connection with the studies on milk powder to study the influence of various levels of casein on growth, reproduction, and the rearing of the young. Table III summarizes the data obtained. The figures are self-explanatory. The diets contained butter fat 5 per cent, salt mixture 3.7 per cent, yeast 5 per cent, casein in various amounts from 15 to 40 per cent, and the remainder of the ration to 100 per cent consisted of dextrin. The yeast contained 46 per cent of protein. 7 young were weaned on 40 per cent of protein. Practically all the young were reared at the 15 per cent level. The mortality was

TABLE III

Results Obtained on Growth, Reproduction, and Rearing of Young with Different Amounts of Casein.

Casein	No of females.	No of males	No of litters	Total No of young	No of young that lived	No of young that died	Curve of growth
<i>per cent</i>							
15	6	2	6	37	36	1	+
20	6	2	5	39	27	12	++
22	5	2	4	28	20	8	++
25	4	2	10	59	41	18	++
30	4	2	6	57	38	19	++
40	4	1	6	39	7	32	+

increased considerably between levels of 20 and 30 per cent. The table shows, furthermore, that the effects recorded by Hartwell are apparent at levels of protein intake below 33 per cent. The mortality of the young is high even though the first generation is given the diet from the time of weaning to maturity. The data show, furthermore, that it is unnecessary to assume the existence of a new vitamin for reproduction.

Wheat Embryo as a Source of Vitamin B and Its Relation to Growth, Reproduction, and the Rearing of the Young.

Evans and Bishop (1) showed that diets containing yeast as the only source of vitamin B when fed to rats resulted in the production of few or no young. Successful results were obtained

when the yeast was replaced by wheat embryo. The conclusion was drawn by the above investigators that wheat embryo contains the dietary substance X, and that yeast is entirely deficient or contains the substance in limited quantity.

The following experiments were performed on rats of the same age and weight as in the previously related work on milk.

TABLE IV.

Data Illustrating Relation of Quantity of Wheat Embryo in Diet to Growth, Reproduction, and Rearing of Young.

Wheat embryo.	No. of males.	No. of females.	No. of litters.	Total No. of young born.	No. of young that died.	No. of young that were reared.	No. of young consumed.	Young that died for lack of care by mother.	Young that died because mothers died.	Curve of growth.
<i>per cent</i>										
1.5	3	3	0	0	0	0	0	0	0	—
2.0	5	8	0	0	0	0	0	0	0	—
2.5	7	5	4	25	11	0	8	0	6	—
3.5	3	3	3	15	15	0	0	0	0	—
4.0	2	5	4	28	22	0	6	0	0	+
4.5	3	3	2	12	12	0	0	0	0	+
5	15	21	21	126	108	0	6	6	6	+
6	1	3	5	38	38	0	0	0	0	++
7	10	16	11	79	56	23	0	0	0	++
8	4	6	9	65	6	59	0	0	0	+++
9	1	3	2	17	0	17	0	0	0	+++

The diets consisted of purified casein 18 per cent, filtered butter fat 5 per cent, salt mixture 185, 3.7 per cent, wheat embryo in various amounts from 1.5 per cent up to and including 9 per cent, and the remainder of the diet to 100 per cent was composed of dextrin. The casein was washed for several weeks with distilled water, acidified with acetic acid until all vitamin B had been removed. Some casein was extracted with 95 per cent ethyl alcohol in a large continuous extractor similar to a Soxhlet apparatus. No casein was employed which did not show the absence of vitamin B in so far as that is possible by nutrition experiments. Dextrin was prepared by moistening starch with 1 per cent citric acid solution and autoclaving the mixture at 20 lbs. pressure for 3 hours. The results obtained by the use of various levels of wheat embryo are recorded in Table IV.

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The figures show that when wheat embryo is employed as the sole source of vitamin B results are obtained which are very similar to those secured with yeast as the only source of this vitamin (4). The similarity of the data obtained with wheat embryo and yeast offers additional evidence that it is not necessary to postulate the existence of a new vitamin for reproduction. The mortality of the young is 100 per cent at and below the 6 per cent level when wheat embryo is used as the only source of vitamin B in the diet. When wheat embryo is employed at higher levels the number of young which is reared is directly proportional to the amount of wheat embryo used. The young which were not reared died in an emaciated condition in periods of time varying from 1 to about 4 weeks. The fact that the young live as long as they do indicates that milk is being secreted and that it is either deficient in quantity or quality or both. Future experiments will reveal whether the results obtained are to be ascribed to difference of level of vitamin B or to a new dietary factor, the nature of which is unknown.

SUMMARY.

1. Normal growth and reproduction result when whole milk powder is the only source of protein and vitamins in the diet.
2. The mortality of the young on many of the diets recorded is high.
3. In order to obtain optimum results in nutrition the proportions of fat, protein, and salts must be within certain limits.
4. The proportion of protein in the diet has a marked influence on the well-being of the suckling, although the first generation may grow at the normal rate.
5. The data show that it is unnecessary to assume the existence of a new vitamin for reproduction.

Thanks are due Professor V. E. Nelson for suggesting the work and for advice during its progress. Acknowledgement is also made to Mr. V. G. Heller for assistance in the collection of some of the data. The author desires to thank the Merrell-Soule Company for supplying the milk powders and The Fleischmann Company for the yeast. When this work was completed and ready

for publication an article appeared in the December, 1923, number of the Journal of Biological Chemistry by A. H. Smith and E. Carey which discusses certain of the points referred to in this paper.

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THE CHEMISTRY OF JAFFÉ'S REACTION FOR CREATININE. A RED TAUTOMER OF CREATININE PICRATE.

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Considering the length of time that has elapsed since Jaffé (1) reported his well known reaction for creatinine and considering, also, the widespread use that has been made of this reaction since Folin (2) employed it as the basis of his colorimetric determination of creatinine, it is rather surprising that so little is known of the chemical changes involved.

According to Chapman (3), the red color is due to the formation of the sodium salts of picramic acid (monoamino-dinitrophenol) and diamino-nitrophenol. But, apparently, the only foundation for this statement was the fact that he was able to match the color of the creatinine reaction product with a mixture of these substances. The color of solutions of the sodium salt of diamino-nitrophenol is so much more intense than that of solutions of sodium picramate and is of so different quality that the explanation is inherently improbable, for it is difficult to understand how, if the red color is due to a mixture of these two reduction products, mixtures of creatinine, picric acid, and alkali in such widely different proportions could give colors of the same quality.

Several years ago, Dr. S. R. Benedict found that hydrochloric acid precipitated a red substance from a concentrated mixture of picric acid, excess of creatinine, and sodium hydroxide. At his suggestion, Mr. H. W. Banks, 3rd, then of the staff of this laboratory, prepared a considerable quantity of this substance and attempted to study its properties. Shortly after beginning this work, Mr. Banks entered military service and the work was abandoned. However, a small quantity of this "red substance" remained in the laboratory.

Comparatively recently, in the course of other work, some crystals were obtained by adding acetic acid to a mixture known to contain, among other substances, creatinine, picric acid, and sodium hydroxide. These crystals gave a red color with picric acid and sodium hydroxide, but melted at 252° , considerably above the melting point of creatinine picrate, $212-213^{\circ}$. The substance was yellow and seemed to be a picrate. Precipitation with nitron showed it to contain 75.2 per cent picric acid, half of which was titratable with sodium hydroxide, with phenolphthalein as indicator. The end-point was not changed by the addition of formaldehyde, indicating the absence of free amino groups. The total nitrogen content was 20.6 per cent. These results seemed to indicate that it was a dipicrate of a substance derived from creatinine by oxidation or by the addition of water. The same substance was obtained by the acidification of a mixture of creatinine, picric acid, and sodium hydroxide, so that the cooperation of the other substances present at the time of the original preparation was excluded.

It was then decided to determine the rate of destruction of picric acid in the Jaffé reaction. Accordingly, a mixture of 50 cc. of a solution of creatinine zinc chloride in 0.1 N hydrochloric acid, 75 cc. of an approximately 1 per cent picric acid solution, and 25 cc. 10 per cent sodium hydroxide was prepared. At intervals of 1, 5, 10, and 30 minutes and of 1, 3, 6, and 24 hours, 5 cc. samples were removed, diluted to 100 cc. (or to smaller volume when necessary), and compared in the colorimeter against 0.5 N potassium dichromate. At the same intervals, other samples of 10 cc. each were acidified with acetic acid, heated to boiling, and precipitated with nitron (4); the nitron picrate filtered on a Gooch, washed, dried, and weighed. A control experiment, in which water was used instead of the creatinine solution and in which only the picric acid content was determined, was also performed. The results are summarized in Table I. It is evident that during the period of development of the color, there was no loss in picric acid. It was only after the color began to fade, or after at least 30 minutes, that the picric acid content of the mixture containing creatinine began to diminish and that other colored substances, not precipitated by nitron, began to be formed. In the control, the rate of destruction of picric acid and of formation of other

colored substances was very much slower, indicating that the creatinine played a part in the destruction of the picric acid.

The failure to observe a destruction of picric acid within the first 30 minutes was so surprising that the experiment was repeated, using a smaller amount of picric acid, in order that a slight reduction should be more readily apparent. The mixture contained 0.100 gm. of pure creatinine, 200 cc. of water, 50 cc. of 1 per cent picric acid solution, and 50 cc. of 10 per cent sodium hydroxide. The results are summarized in Table II. In spite of the reduced amount of picric acid, practically the full color

TABLE I.

Rate of Color Development and Picric Acid Destruction in a Mixture of Creatinine, Picric Acid, and Sodium Hydroxide.

Time.	Creatinine in 5 cc.	Picric acid in 10 cc.	Color of filtrate from nitron picrate.
<i>min.</i>	<i>mg.</i>	<i>mg.</i>	
1	1.80	49.2	Colorless.
5	1.82	49.1	"
10	1.81	48.2	"
30	1.82	48.1	Very pale yellow.
<i>hrs.</i>			
1	1.80	49.0	" " "
3	1.62	47.8	Pale yellow.
6	1.31	46.8	" "
24	0.477	39.9	Yellow.
Control without creatinine.			
4 min.		48.9	Colorless.
6 hrs.		48.7	Very pale yellow.
24 "		48.2	" " "

value was obtained (1.64 mg. of creatinine in 5 cc. instead of 1.67 mg.). Again, there was no apparent destruction of picric acid until the color began to fade.

The amount of picric acid was still further reduced. A mixture of 100 mg. of creatinine in 225 cc. of water, 25 cc. of 1 per cent picric acid, and 50 cc. of 10 per cent sodium hydroxide was prepared and analyzed at intervals as in the preceding experiments. The results, summarized in Table III, again show that there was no destruction of picric acid until after the color had begun to fade. They also show that, with so little picric acid, the creatinine did not develop its full chromogenic value.

TABLE II.

Rate of Color Development and of Picric Acid Destruction in a Mixture of Creatinine, Picric Acid, and Sodium Hydroxide.

Time.	Creatinine in 5 cc.	Picric acid in 25 cc.	Color of filtrate from nitron picrate.
<i>min.</i>	<i>mg.</i>	<i>mg.</i>	
1	1.34	40.8	Colorless.
5	1.64	41.0	"
10	1.64	41.0	"
30	1.65	40.8	"
<i>hrs.</i>			
1	1.62	40.5	Very pale yellow.
3	1.40	38.6	Pale yellow.
6	1.06	35.7	" "
24	0.31	24.2	Yellow.
Control without creatinine.			
4 min.		40.7	Colorless.
6 hrs.		40.5	Very pale yellow.
24 "		40.2	" " "

TABLE III.

Rate of Color Development and of Picric Acid Destruction in a Mixture of Creatinine, Picric Acid, and Sodium Hydroxide.

Time.	Creatinine in 5 cc.	Picric acid in 25 cc.	Color of filtrate from nitron picrate.
<i>min.</i>	<i>mg.</i>	<i>mg.</i>	
1	0.921	20.6	Colorless.
5	1.24	20.5	"
10	1.25	20.4	"
30	1.24	20.3	Very pale yellow.
<i>hrs.</i>			
1	1.21	20.0	" " "
3	1.03	18.9	Pale yellow.
6	0.579	14.4	Yellow.
24	0.135	9.66	"
Control without creatinine.			
4 min.		20.3	Colorless.
6 hrs.		20.0	"
24 "		19.9	Very pale yellow.

In this experiment, there were present 2.5 mg. of picric acid for each milligram of creatinine, or 1.23 mols of picric acid for each mol of creatinine. Inasmuch as 75 per cent of the full chromogenic value of the creatinine was developed, it is probable that only 1 mol of picric acid actually enters into the reaction, but that the reaction is not complete unless the picric acid is present in considerable excess. It is apparently complete when there are 2.5 mols of picric acid for each mol of creatinine.

It might be objected that the formation of a precipitate with nitron, that is insoluble in hot, dilute acetic acid or hot water, is not a sufficiently specific test for picric acid. Accordingly, a mixture of 0.100 gm. of creatinine, 50 cc. of 1 per cent picric acid, and 50 cc. of 10 per cent sodium hydroxide was allowed to stand 5 minutes and was then strongly acidified with hydrochloric acid and extracted with benzene in a continuous extraction apparatus until colorless. The benzene was distilled from the extract and the residue was dried at 100° to constant weight. There were obtained 0.5016 gm.; calculated 0.5000 gm. The substance melted at 122°.

0.1016 gm.	yielded	18.0 cc.	nitrogen at	26.0°	and	756 mm.	or	18.6 per cent.
0.1061 "	"	"	10.9 "	"	"	24.0°	"	762 "
								18.5 "

Calculated for picric acid, 18.4 per cent.

The substance was unquestionably picric acid.

It is evident that the original quantity of picric acid can be recovered from the mixture of creatinine, picric acid, and sodium hydroxide. Is the same true of the creatinine? To answer this question, the extracted liquid in the experiment just described was evaporated until crystals appeared and was then treated with alcohol. The filtrate was again evaporated and the treatment with alcohol was repeated until no precipitate appeared on the addition of absolute alcohol. The alcohol was evaporated and the residue was dissolved in water and used for a few tests, including the preparation of a picrate. This melted at 142° and contained 73.6 per cent of picric acid. It was not so recognized at the time, but later experiments showed that it was probably an impure creatinine picrate. Proof of the recovery of creatinine will be furnished later.

In other somewhat similar experiments, attempts were made to precipitate the creatinine as the zinc chloride compound. These were not successful but, as the addition of pure creatinine to the resulting mixtures did not produce a precipitate, they were inconclusive.

Reference has been made to the yellow crystals obtained on acidifying the mixture of creatinine, picric acid, and sodium hydroxide. Repetitions of the experiment gave inconsistent results, sometimes crystals were obtained, but at other times, under apparently the same conditions, they were not.

In order to help establish the nature of the base, an attempt was made to prepare the hydrochloride. 1.1265 gm. of the picrate were dissolved in hot water, 5 cc. of concentrated hydrochloric acid were added, and the mixture was extracted with benzene, in a continuous extraction apparatus, until colorless. The extract was dried and weighed. The yield was 0.8410 gm. or 74.6 per cent of picric acid. The extracted liquid was evaporated and treated with absolute alcohol until separated into two fractions, one of which was soluble in absolute alcohol while the other was not. The presence of potassium was suspected. The insoluble portion yielded 0.4182 gm. of sodium potassium cobalti-nitrite, which is equivalent to 0.0720 gm. of potassium, or 6.39 per cent of the original picrate. The soluble portion was evaporated to dryness. It weighed 0.2472 gm. or 21.7 per cent. Treatment with sodium picrate yielded a picrate, melting at 213°. Of this, 0.0909 gm. yielded 0.1431 gm. of nitron picrate, the equivalent of 66.7 per cent picric acid. Creatinine picrate melts at 212–213° and contains 67 per cent of picric acid.

	Picric acid.	Potassium.	Creatinine hydrochloride.
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Calculated for creatinine potassium picrate.....	75.2	6.41	24.5
Found.....	74.6	6.39	21.7

The correspondence in picric acid and potassium content and the isolation of creatinine picrate leave little doubt but that the original substance was creatinine potassium picrate. The low value for creatinine hydrochloride was probably due to partial

dissociation. The identity of the substance was further established by the preparation of pure creatinine potassium picrate from urine, by repeated crystallization from water and from alcohol. It had the same melting point, 247–252°, depending upon the rate of heating, and the same property of further liberation of gas at about 270°.

The potassium in the picrate could have come only from the sodium hydroxide used. Tests showed that this, a commercial "by alcohol, C.P." grade contained potassium. When pure sodium hydroxide was used, no crystals were obtained. Thus, 0.440 gm. of creatinine zinc chloride was dissolved in 100 cc. of hot 1 per cent picric acid solution, diluted with 200 cc. of water, and then cooled. There were then added 100 cc. of 10 per cent sodium hydroxide (from sodium) and, after 5 minutes, enough glacial acetic acid to make the mixture acid. The mixture was kept in the ice chest but no crystals had appeared by the following day. 1 cc. of a saturated solution of potassium chloride was added. Crystals appeared almost immediately. These were filtered out and recrystallized from hot water. After filtering and drying, these weighed 0.6094 gm. The volume of the combined filtrates was 520 cc. If they contained as much creatinine potassium picrate as was dissolved in water at the temperature of the laboratory, about 15°, or 0.141 gm. per 100 cc., there would have been 0.733 gm. in solution, giving a total yield of 1.34 gm. The calculated yield was 1.18 gm. The crystals melted at 247° and gave off more gas at about 270°. 0.0917 gm. yielded 0.1613 gm. of nitron picrate, the equivalent of 74.5 per cent picric acid. The filtrate was freed of nitron with nitric acid and, after adding a little sulfuric acid, was evaporated and ignited. There were obtained 0.0129 gm. of potassium sulfate, the equivalent of 6.32 per cent potassium. The objection that the creatinine potassium picrate obtained in the earlier experiments might have been obtained from a small quantity of creatinine which did not enter into Jaffé's reaction is thus disposed of and the question as to the quantitative recovery of the creatinine is answered in the affirmative.

Reference has already been made to the red substance obtained by Benedict and by Banks on adding hydrochloric acid to a warm, concentrated solution of sodium picrate, sodium hydroxide, and

excess creatinine. This substance turned yellow at about 130° and melted at about 190° . 0.1195 gm. yielded 0.1698 gm. of nitron picrate, corresponding to 60.2 per cent picric acid. The filtrate was yellow, indicating the presence of some reduction product of picric acid. Extraction of the original substance with hot benzene removed no picric acid, indicating that all was combined. 0.1472 gm. was heated with silver nitrate and nitric acid. 0.0017 gm. of silver chloride was obtained. The filtrate was freed of silver with hydrochloric acid and the filtrate, after adding sulfuric acid, was evaporated and ignited. The residue weighed 0.0012 gm. When dissolved in water and treated with sodium cobalti-nitrite solution, it gave no precipitate, indicating the absence of potassium. 0.0017 gm. of $\text{AgCl} = 0.00069$ gm. of NaCl and 0.0012 gm. of $\text{Na}_2\text{SO}_4 = 0.00099$ gm. of NaCl . Apparently, the sample contained about 0.001 gm. or 0.07 per cent NaCl .

Another sample of 0.5308 gm. was heated at 139° (xylene vapor), *in vacuo*, for 4 hours. It turned brown within 30 minutes. The loss in weight was 0.0128 gm., or 2.41 per cent. The residue was dissolved in hot water. The small quantity of insoluble material was filtered out, dried, and weighed. It amounted to 0.0063 gm., or 1.87 per cent. The solution was made acid to Congo red with hydrochloric acid and was extracted with benzene in a continuous extraction apparatus. The dried extract weighed 0.3136 gm. When dissolved in water and titrated with methyl red as indicator it required 13.2 cc. of 0.1016 N NaOH , which is the equivalent of 0.3073 gm. of picric acid. The neutralized solution was diluted to 300 cc. Portions of 50 cc. each yielded 0.1204 and 0.1208 gm. of nitron picrate, or 0.3065 gm. of picric acid in all. The picric acid content of this sample was, therefore, 57.8 per cent. The acid liquid was extracted with ether. The extract weighed 0.0094 gm., but when dissolved in hot water gave no precipitate with nitron. The acid solution was now extracted with ligroin. The extract weighed 0.0064 gm. and contained no picric acid. The acid liquid was still brown. It was evaporated to small volume and this evaporation was repeated several times to drive off most of the hydrochloric acid. It was finally neutralized, filtered from a small quantity of insoluble material, and treated with 70 cc. of a 1 per cent picric acid solution and 0.5 cc.

of saturated potassium chloride solution. The crystals obtained were recrystallized from hot water. They weighed 0.220 gm. and melted at 247°. Upon analysis, 0.0987 gm. yielded 0.1741 gm. of nitron picrate. Found 74.7 per cent picric acid. Calculated for creatinine potassium picrate, 75.2 per cent. 0.1205 gm. yielded 0.0163 gm. of potassium sulfate. Found 6.07 per cent potassium. Calculated, 6.41 per cent.

The volume of the combined mother liquids was 120 cc. which could have dissolved 0.169 gm. of creatinine potassium picrate, indicating a total yield of 0.389 gm., equivalent to 0.0722 gm., or 13.5 per cent, creatinine in the original "red substance." This amount of creatinine would account for 27.55 per cent picric acid in combination as creatinine picrate.

Portions of 0.0350 gm. each of this "red substance" were analyzed for creatinine by Folin's method, using the potassium dichromate standard. The readings indicated an apparent content of 0.00938 gm. of creatinine, or 26.8 per cent. But, since the calculations given above indicate a much smaller quantity, it seems that part of the color was due to reduction products of picric acid. Summarizing the results, it appears that the "red substance" obtained by Banks on adding hydrochloric acid to the warm, concentrated mixture of sodium picrate, excess of creatinine, and sodium hydroxide consisted of

	<i>per cent</i>
H ₂ O.....	2.41
NaCl.....	0.07
Insoluble material.....	1.87
Creatinine.....	13.5
Picric acid combined with creatinine.....	27.55
Picric acid, otherwise combined.....	30.25

The remainder, or 24.4 per cent, probably consisted of reduction products of picric acid and, possibly, of decomposition products of creatinine.

With the knowledge obtained from these experiments, it was a simple matter to obtain, in a fairly pure condition, the red substance responsible for the color. This was best done by dissolving creatinine picrate and an equal amount of picric acid (2.5 mols of picric acid for each mol of creatinine) in hot water, allowing the mixture to cool to room temperature, and then adding enough

10 per cent sodium hydroxide to dissolve all the crystals and a slight excess. After 5 minutes, the mixture was acidified by the addition of concentrated hydrochloric acid. The precipitate was filtered out and washed with water until the filtrate was free of chlorides, then with alcohol and ether. After drying *in vacuo* over concentrated sulfuric acid, it was found to still contain free picric acid. This was removed with benzene. It then formed a brilliant red powder. It contained 65.0 per cent picric acid. When heated at 139°, *in vacuo*, for 8 hours, 1.6502 gm. lost 0.0295 gm. A little yellow deposit was observed on the cooler parts of the drying apparatus. This was dissolved out, precipitated with nitron, and weighed. The amount obtained corresponded to 0.0026 gm. of picric acid. Apparently, not quite all the free picric acid had been removed by the extraction with benzene. Correcting for the picric acid, the loss in weight becomes 0.0269 gm., or 1.60 per cent, water. This is less than 1 mol of water for each 2 mols of picric acid or creatinine and probably represents adsorbed, rather than chemically combined, water. The residue in the drying apparatus melted at 210–212°. Creatinine picrate melts at 212–215°. It was dissolved in hot water and added to a solution of 1.288 gm. of potassium picrate. The crystals that separated on cooling were recrystallized from hot water, filtered, and dried. They weighed 2.3475 gm. The 171 cc. of combined filtrates could dissolve 0.241 gm. of creatinine potassium picrate, giving a total yield of 2.589 gm. Calculated, 2.938 gm. The crystals melted at 247°.

0.1105 gm. yielded 0.1944 gm. nitron picrate and 0.0159 gm. K_2SO_4 .

Found. 74.5 per cent picric acid and 6.46 per cent potassium.

Calculated. 75.2 " " " " " 6.41 " " "

The proof of the identity of the heated substance with ordinary creatinine picrate would appear to be complete. The red substance precipitated by hydrochloric acid from the mixture of creatinine, excess of sodium picrate, and sodium hydroxide can only be a tautomeric form of creatinine picrate.

In connection with quite another problem, an attempt had been made some time previously to determine with what substances more or less related to picric acid, a reaction similar to Jaffé's could be obtained. The substances used were *o*-nitrophenol,

p-nitrophenol, 2, 4-dinitrophenol, 3, 5-dinitrophenol, trinitro-*m*-cresol, 1, 3, 5-trinitrobenzene, 2, 4, 6-trinitrotoluene, 2, 4-dinitrobenzoic acid, 3, 5-dinitrobenzoic acid, 2, 4, 6-trinitrobenzoic acid, 3, 5-dinitrosalicylic acid, 3, 5-dinitro-*p*-cresol, and picramic acid. With both trinitrotoluene and trinitrobenzoic acid, using concentrations that did not give too intense a color on the addition of sodium hydroxide, this color was slightly intensified by the presence of creatinine. With trinitrobenzene, the intensification was greater, but still very slight. With the other substances, no effect of the addition of creatinine was observed.

SUMMARY.

In Jaffé's reaction for creatinine, only 1 mol of picric acid appears to be required for each mol of creatinine, although the reaction is not complete unless a considerable excess is present.

If the alkaline mixture, made up in the proportions recommended by Folin in his original method for the determination of creatinine, is reacidified within 10 minutes, both picric acid and creatinine may be quantitatively recovered. After standing more than 30 minutes, the color of the alkaline mixture begins to fade and the picric acid can no longer be quantitatively recovered.

By the addition of hydrochloric acid to a concentrated mixture of creatinine, excess of sodium picrate, and slight excess of sodium hydroxide, a red precipitate was obtained. After washing and drying, this formed a brilliant red powder. When heated to 139°, it was transformed into ordinary creatinine picrate. Apparently, it is a tautomeric form of creatinine picrate. It is the formation of this substance that appears to be responsible for the red color in Jaffé's reaction for creatinine.

The reaction is not given by a number of other substances more or less similar in constitution to picric acid. A slight intensification of the colors of alkaline solutions of the salts of trinitrobenzene, trinitrotoluene, and trinitrobenzoic acid was observed.

We are indebted to Dr. S. R. Benedict and to Mr. H. W. Banks, 3rd, for permission to use the red substance prepared by them.

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A NOTE ON RUBIDIUM AND CESIUM CREATININE PICRATES.

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New York.)

(Received for publication, February 15, 1924.)

In 1886, Jaffé (1) described creatinine potassium picrate. The slight solubility of this substance has made it useful in the preparation and identification of creatinine. However, it is too soluble to permit of its use in the isolation of very small amounts of creatinine from large quantities of biological material. It seemed to be advisable to attempt to prepare other double picrates of creatinine.

The hot solutions of creatinine, picric acid, and the sulfate or chloride of the metal or radicle were mixed in equivalent quantities and a little sodium acetate was added. After cooling, the crystals obtained were filtered out and recrystallized from hot water. With lithium and sodium, only creatinine picrate, identified as such by melting point and picric acid content, was obtained. With ammonium picrate and trimethyl ammonium picrate, mixtures of these with creatinine picrate were obtained. However, on recrystallizing a few times, pure creatinine picrate was isolated. Definite double salts were obtained with rubidium and cesium. These were analyzed for picric acid content by precipitation with nitron (2), and for the metal by evaporation with concentrated sulfuric acid and ignition.

Creatinine rubidium picrate, yellow needles, M. P. 256–257°. 0.1070 gm. yielded 0.1770 gm. nitron picrate.

Found.	70.1	per cent	picric acid.
Calculated.	70.0	"	" " "

0.6471 gm. yielded 0.1303 gm. Rb_2SO_4 .

Found.	12.9	per cent	Rb.
Calculated.	13.05	"	" "

Creatinine cesium picrate, yellow needles, M. P. 255°.

0.1019 gm. yielded 0.1571 gm. nitron picrate.

Found. 65.3 per cent picric acid.

Calculated. 65.2 " " " "

0.5048 gm. yielded 0.1270 gm. Cs_2SO_4 .

Found. 18.45 per cent Cs.

Calculated. 18.9 " " "

The molecular solubility of these substances is considerably less than that of creatinine potassium picrate. Thus, at about 10°, per liter of saturated solution, there were dissolved, of

Creatinine potassium picrate, 1.41 gm., or 0.00231 M.

" rubidium " 0.960 " " 0.00146 M.

" cesium " 1.18 " " 0.00168 M.

It is planned to use the rubidium salt in an attempt to isolate creatinine from blood.

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ANALYSIS OF THE GASES OF THE AIR-BLADDER OF THE CALIFORNIA SINGING FISH, PORICHTHYS NOTATUS.

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(Received for publication, March 1, 1924.)

Analyses of the gases of the air-bladders of many fishes have been reported at intervals during the past century. The intrinsic interest of the problem is in large measure due to the assumption that the air-bladder mechanism is an apparatus for static control, especially in fishes that migrate vertically in the sea.

Morphologically the air-bladder of fishes is comparable to the lung of higher vertebrates. In fact, in many fishes there is an opening between the air-bladder and the pharynx through which air is taken or expelled; for example, the fresh water *Lepidostidæ*. In many others, as in the present genus, there is no connection. The cavity, although derived by a pharyngeal evagination, has been cut off in an early embryological stage and is a closed chamber in the adult.

The discovery first reported by Biot (1) that the gases of the air-bladders of deep sea fishes contain oxygen far in excess of the oxygen of atmospheric air, introduced a new interest in the subject. It was easy to explain the excess of nitrogen commonly observed in the air-bladders of surface fishes. But an excess of oxygen over the atmospheric percentages cannot be explained on the usual physical partial pressures and diffusion rates between the gases of the blood and the air-bladder cavities.

Bohr (2) made a classic series of analyses on *Gadus callarias* which has a large air-bladder and no "air passage," or open duct. When the fishes were in equilibrium the air-bladder gases contained from 8.2 to 19.6 volumes per cent of oxygen. Only an occasional exception had over 21 volumes per cent of oxygen in the first sample drawn. Specimens from a depth of 14 meters were bloated from gas expansion and swam on their backs when brought to the surface. The gas analyzed immediately contained 52 volumes per cent oxygen, but by the next day the distention had disappeared

and the oxygen was reduced to from 10 to 16 per cent in a half dozen specimens. When Bohr analyzed a series of samples from the same fish the first sample was low in oxygen, but succeeding samples were above atmospheric air in oxygen content, an observation previously made by Moreau (3). Bohr showed that the air-bladder did not fill with gas after emptying if the abdominal branches of the vagi were first cut.

Woodland (4) has published a number of figures showing the histological structure of the so called red bodies or gas glands and of the rete mirabile. Intracellular vacuoles of the gland cells he interpreted as gas vacuoles. Woodland advocates a modified secretory theory of gas formation, but it does not appear that he has clarified the facts beyond the simple hypothesis of Bohr stated in 1894 as follows: "We must therefore ascribe to the cells of the walls of the air-bladder the power of secreting oxygen."

This secretory hypothesis of gas separation in fishes has been generally accepted as accounting not only for the presence of gas but for the unusual percentage of oxygen in the gases of the closed type of air-bladder. The secretory hypothesis was suggested by Bohr (5) as applicable in the transference of oxygen through the walls of the alveoli of the lungs of vertebrates.

The application of the hypothesis of pulmonary oxygen secretion to man during oxygen distress was first advocated by Douglas and Haldane (6). Later Douglas, Haldane, Henderson, and Schneider (7) gave extensive data tables in their Pikes' Peake investigations in support of this hypothesis. The inherent improbability of these results for man has led to experimental data by Hartridge (8), Barcroft and coworkers (9), and Greene and Greene (10), all failing to confirm the hypothesis. In fact, the difficulty seems to be explained by misplaced reliance by Douglas and Haldane on the carbon monoxide method. It is also questionable whether data from the very specialized fish air-bladder can be assumed to be directly applicable for the homologous lungs of mammals, although physiological literature abounds in examples of this type of reasoning.

The California singing fish yields data of value in this connection. This fish is present in the Pacific coast waters from California to Alaska and is numerous at Monterey Bay. They are from 10 to 20 inches long and in shape and coloration much the same as the better known Mississippi mud-cat. The air-bladder in *Porichthys* is closed off in early embryological development. In the adult it consists of a double chamber. It is about 50 mm. long by 45 mm. broad in a 25 cm. fish. It is U-shaped, each limb about the size of a glove finger-tip. Across the base of the cavity of the U there is a membrane formed like a drum head, but with a tiny opening in the center. This is primarily a noise-producing apparatus, depending upon variations in pres-

sure accomplished by the strong muscles attached to the sides of the sac. When the gas is withdrawn from an air-bladder, the cavity is refilled by the activity of the so called rete mirabile and of the red bodies, the gas glands. The red bodies in *Porichthys* are oval plaques about 1 mm. thick, 3 to 6 mm. in diameter, and from twelve to fifteen in number.

Method of Gas Analysis.

The gases of the *Porichthys* air-bladder were analyzed by the Haldane air analysis methods. Samples of the gas were drawn from the air-bladder into a Haldane gas pipette over mercury. The pipette was the usual form provided with double stop-cocks. Long, steel needles, Luer's form, size 18 or 20 with a length of 2 inches or more, were connected to the pipette by heavy walled, pure gum, capillary gas tubing. New needles were carefully oiled with liquid petrolatum before use to prevent amalgamation.

A fish-holder of wood was devised, so shaped that a specimen could be bound quickly and firmly, yet with the belly exposed and the gills free to move in respiration when returned bound to the aquarium. This holder supported the fish as in an inverted floating dock. When all was prepared one quickly lifted the fish out of the aquarium and placed the holder on the table with the abdomen of the fish up. The gas pipette was filled with mercury to the tip of the needle, the cock closed, and the needle inserted through the abdominal wall into the cavity of the air-bladder. The gas was then drawn under moderate negative pressure and the fish returned to the water. The tough skin and thick, connective tissue wall of the air-bladder are safe seals against the negative pressures used. The volume of gas drawn was all the cavity would yield, from 2 to 6 cc. from most fishes.

Since the gas samples available were small, the first analyses were made by adding the samples to the ordinary Haldane pipettes, starting with 7 cc. or more of nitrogen, which is too large a dead space. These analyses quickly revealed the great excess of oxygen in the air-bladder gases. The Haldane pipette was then replaced by the micro pipette of the Van Slyke amino nitrogen apparatus, the capillary section of which has a volume of

2 cc. and the bulb, to 10 cc. The dead space of the apparatus with this assembly was very much less and duplicate analyses checked satisfactorily. However, the fractional absorption was slow and it was necessary to wash the gas 75 or 100 times to secure constant readings.

TABLE I.

Air-Bladder Gases of the Phosphorescent Fish, Porichthys notatus.

Fish No.	Date.	Gas drawn	Sample.	O ₂	CO ₂	N ₂
	1923	cc.	cc.	per cent	per cent	per cent
1	July 6	4	3 32	51 5	0.3	4.8
	" 9		1.60	62.0	0.0	38.0
2	" 11		2 94	26 9	1.3	71.7
3	" 12	20	2 42	82.6*	1.5	15.9
	" 13	100	2 92	81 1	0.7	18.2
4	" 18		3 33	71 7	2.4	25.8
	" 28		0 89	84 7	1.8	13.5
	" 30		1 42	83 9	0.0	16.1
5	" 28	10	2 00	80 2	1.2	18.6
	" 31	2	1 49	70 1	0.4	29.4
	Aug. 7	6	2 00	66 2†	0 0	33.8
	" 13	4	2.00	31.3‡	0.1	68.6
	" 13	;	1 97	58.8	1.1	41.1
	" 16	1.2	2 00	68 1	1.6	30.3
	" 28	4	1 50	35.9	0 3	64.8
6	" 9	1	0 93	30.0	Trace.	70.0
7	" 2	2	1.54	49 3	1 9	48.8
8	" 8	1	0.98	88 2	0.4	11.4

* Overdistended with gas and floating on surface.

† Last sample before inflating with atmospheric air.

‡ First sample after atmospheric air.

Analytical Results.

Only a short series of fishes was available. But one of these in particular was observed through a period of 30 days and with variations in the conditions. The total data are sufficient to establish the fact that the gas in the air-bladder of this species is always high in oxygen, very low in carbon dioxide, and comparatively low in residual nitrogen. The carbon dioxide was usually low and inconstant, 0 to 2 per cent; in one fish in poor condition, 2.4 per cent. These percentages are significant from

the standpoint of the control of formation of the gases in the air-bladder and confirm previous observations.

The oxygen of the air-bladder gas was very high, reaching a maximum of 88.2 per cent in one specimen, and never so low as that in atmospheric air. In fact, the lowest figures in the series were 26.9 and 30 per cent of oxygen, and yet only a trace of carbon dioxide was present.

When the gas was withdrawn from the air-bladder in the process of sampling, the attempt was always to empty the cavity as completely as possible. To do this a negative pressure of 30 to 50 mm. of mercury was sometimes used on the gas pipette together with mechanical pressure and kneading of the air-bladder through the abdominal wall. After withdrawing gas the air-bladder was rapidly filled again, in less than 24 hours, apparently in as short a time as 4 hours in some cases. Fortunately the vocalizing function of the air-bladder assisted in the determination of the rate of refilling with gas. When gas was withdrawn the fish lost the power of vocalizing, a reaction depending on gas pressure in the cavity. It often happened that it produced a series of sounds while the gas was being drawn. It was then easy to distinguish the diminishing intensity and final failure of sound. In a large and noisy specimen, from which gas samples were repeatedly drawn, it was observed that the recovery of the intensity and tone of the sound was progressive, apparently as the air-bladder filled with gas and as tension was reestablished on the vibrating diaphragm. Normal tone was reestablished in about 4 hours. However, gas pressures were not measured.

Bohr has given the evidence already reviewed that in certain fishes the refilling gas has a much higher proportion of oxygen than the first sample of gas drawn. His first samples, with few exceptions, contained oxygen percentages well below normal air. In *Porichthys notatus*, all samples analyzed have oxygen higher than atmospheric air. In the three first samples, the oxygen was 80.2, 82.6, and 88.2 per cent, respectively. In one of these the percentage of oxygen was lower after the first sample; namely, July 28, 80.2; July 31, 70.1; and August 7, 66.2. In two the percentage of oxygen increased, from 51.5 to 62 per cent, and from 71.7 to 83.9 per cent. In one it remained practically constant in the first and second samples. These specimens indicate

that in the refilling gas the percentage of oxygen is maintained relatively high, and that the refilling percentages may deviate in either direction. Bohr gave evidence that gas secretion was under the influence of the vagus nerve. In *Porichthys notatus* the vagus innervates the large air-bladder muscles and supposedly the vascular and glandular structures concerned in gas production. However, opportunity did not arise to make the tests of vagus control.

One large specimen was tested for the ability to regulate an excessively high experimental pressure of gas in the air-bladder. The normal gas was sampled and the residual gas emptied by a free cannula as completely as possible, aided by external pressure and manipulation. Atmospheric air was then injected into the air-bladder under a pressure of over 6 feet of water. The resulting high tension in the air-bladder could be felt through the abdominal wall. The excessive tension had disappeared after 24 hours, and 2 days later a sample of gas showed an oxygen percentage of 31.3. 4 days later the oxygen of the refilled bladder gave a percentage of 58.8, and again, 8 days later, 68.1 per cent. In the drawing of each of these samples the gas was as completely emptied as possible, and after each withdrawal the fish lost the power of vocalizing. The conclusion is obvious, that atmospheric air can be resorbed from the air-bladder in such a way as to leave a preponderance of oxygen. After these artificial conditions, refilling by the usual mechanism of separating the gas from the blood is with a progressively higher percentage of oxygen.

Incidentally, it is not certainly known whether *Porichthys notatus* uses its air-bladder as a static organ. But apparently it does so. The size of the cavity is smaller than that of many of the more predacious coastal fishes; for example, of the *Sebastes* type. It would not, therefore, be so well adapted for static control. One fish was captured at the surface of the bay so distended with gas that it could neither swim progressively nor descend. Yet in the aquarium after deflation it could not be distinguished from other specimens. The gas of this fish contained 82.6 per cent of oxygen and did not change for 24 hours, 81.1 per cent, before deflation. This certainly was an instance of uncontrolled gas secretion. The volume was expanded

at least ten times the normal and more than the total sea depth at the spot would account for. As a matter of fact, *Porichthys* uses its swim-bladder primarily as a vocal organ and is to that extent a singing fish as its name implies, though grunting fish would be a more appropriate name.

SUMMARY.

1. All gas samples of the air-bladder of *Porichthys notatus* under all conditions contain a percentage of oxygen higher than atmospheric air.

2. Refilled gases present no constant and striking variation in oxygen content in comparison with first samples.

3. Nitrogen can be rapidly removed from the air-bladder of this species.

4. The function of the air-bladder in *Porichthys* may be to some degree a static organ, but it is primarily a vocal organ.

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BILE SALT METABOLISM.

I. INFLUENCE OF CHLOROFORM AND PHOSPHORUS ON BILE FISTULA DOGS.

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Where are bile salts produced in the body? Is this production limited to the liver or does this organ merely excrete these interesting substances? It is generally accepted that bile salts are formed in the liver alone, but what is the evidence on which we base this assumption? For many years it was assumed that bile pigments were formed in the liver and could not be formed elsewhere. It is becoming increasingly evident that bile pigments are formed largely outside of the liver and eliminated through the liver. Evidence for this was brought by Whipple and Hooper (9), using a head and thorax circulation. Recent experiments by Mann, Bollman, and Magath (6), using complete liver extirpation, sugar infusion, and experiments lasting 12 to 18 hours, confirm and extend these observations that bile pigments are formed rapidly and in abundance in the circulation outside of the liver. Can we imagine that bile salts in like fashion may be formed outside of the liver and merely eliminated by it? The evidence, though incomplete, is all in favor of the accepted view that bile salts are formed by the hepatic epithelium. We refer for a discussion of these points to papers by Foster, Hooper, and Whipple (3) and to a recent review of this whole subject (8).

The Eck fistula liver is functionally subnormal and this liver excretes much less bile salt than does a normal liver (4). This is good evidence that bile acids are formed by the activity of the hepatic epithelium. Our experiments, tabulated below, give more

support to the accepted view that the liver cell produces all the bile acids in the body. We observe that very small doses of chloroform by mouth may often reduce the content of bile salts in fistula bile almost to zero. Chloroform is a drug which acts specifically upon the liver epithelial cell which obviously is exquisitely sensitive to this poison. We are familiar with the liver necrosis so readily produced by chloroform, but this observed change in the bile salts can be produced by a small dose of chloroform quite incapable of causing any recognizable structural change in the liver epithelium (fat or necrosis). It is hard to escape the conviction that this specific poison given in small doses produces the remarkable change in bile salt output by a specific action upon the liver cell. This is more evidence that the hepatic epithelial cell is concerned in the reaction,—that it does produce the bile salt. There is no evidence in our experiments for any obstruction to the flow through the bile passages. It might be argued that the chloroform causes a temporary paralysis of the liver epithelium which acts only to *eliminate* (not to produce) the bile salts. If this were true we should expect an increased output of bile salts after this inhibition is removed, assuming a possible heaping up of bile salts in the body fluids and tissues, like the familiar reaction of urea in the body following kidney injury and recovery. At present there is no evidence to favor this hypothetical possibility of bile salt formation in body cells outside of the liver, but this point can be conclusively proved only by liver extirpation experiments similar to those of Mann, Bollman, and Magath (6) dealing with bile pigment production.

Method.

The technique of the bile fistula operation, the routine care of these bile fistula dogs, and the method for the quantitative analysis of bile pigments have been described in detail by Hooper and Whipple (5). The method for quantitative analysis of the bile salt or bile acid (taurocholic acid) in dog bile has been described elsewhere (2). The daily routine is important for the continuance of health and activity of these dogs. The dogs are permitted to exercise about 30 minutes in the yard. They are then set up with the binder and collection tube for 30 minutes to permit of free drainage and escape of any excess of night bile. The dogs are then

brought into the laboratory at about 10.30 a.m. and set up for the standard 6 hour collection. All collection periods are 6 hours unless otherwise stated. Dogs are fed 2 hours after the set up unless note is made to the contrary. After the collection is completed the binders are removed, the dogs are allowed to exercise in the yard, are then put in cages, and are given the night feeding.

The *carbohydrate diet* used in these experiments is made up of boiled potatoes 610 gm., boiled or steamed rice 520 gm., and skim milk 500 cc. About 100 or more calories per kilo of body weight were given per day. The morning and evening feedings were of approximately equal amounts. The chloroform was given by stomach tube in a starch emulsion or by inhalation using an open cone. Light surgical anesthesia was maintained and never pushed to deep narcosis. Phosphorus dissolved in olive oil was given by hypodermic into the muscles and subcutaneous tissues.

EXPERIMENTAL OBSERVATIONS.

The tables given in this paper furnish certain data on chloroform and phosphorus experiments. Many other experiments have been performed, but it is not necessary to publish them all as the examples given are characteristic of the group and the reaction is surprisingly uniform.

Table I gives two experiments on different animals in which a small dose of chloroform by mouth caused a striking decrease in the daily output of bile acid. We note a decrease in volume and pigment excretion and a marked decrease in taurocholic acid. In spite of no clinical abnormality the liver is temporarily disturbed to such a degree that its bile salt output falls to 10 per cent of normal in the second experiment and almost to zero in the first. Within 3 to 4 days we expect a return to normal. During all this time there is no clinical evidence of abnormality,—no loss of appetite, activity, or general condition. This is in striking contrast to experiments given below where we note less disturbance in bile acid secretion with severe or even fatal intoxication (phosphorus and proteose poisoning).

Table II gives two experiments with chloroform anesthesia—light surgical anesthesia lasting 1 hour and $\frac{1}{2}$ hour, respectively. The first experiment shows a slight decrease in bile salt excretion

on the day of chloroform administration but a considerable fall on the next day. The concentration per 1 cc. falls from 0.309 to 0.058 mg. On the following day the level of excretion is practically back to normal. The second experiment on the same dog 1 month later shows a slight reaction to chloroform anesthesia of only $\frac{1}{2}$ hour duration. This experiment, too, illustrates a frequent observation that the 1st day's collection is abnormally high. This is to be explained in part as follows: This collection is made

TABLE 1.
Chloroform by Mouth. Inhibition of Bile Salt Excretion.

Dog. No.	Date.	Vol- ume.	Amino nitrogen.		Tauro- cholic acid in 6 hrs.	Bili- rubin in 6 hrs.	Weight.	Remarks.
			In 1 cc. bile.	In 6 hrs.				
21-60	May 2	65	0.228	14.82	540	83 4	17.0	Carbohydrate diet.
	" 3	49	0.155	7.59	279	87 5	16.8	
	" 4	35	0.126	4 41	162	68.5	16.5	Chloroform (1.5 cc.) in starch 2 hrs. after collection began.
	" 5	8	Tr.	Tr.	Tr.	5 2	16.5	
	" 6	19	0.028	0.53	20	12.1	16.8	No clinical abnormali- ties.
21-45	" 23	29	0.299	8.70	319	20.9	12.5	Carbohydrate diet.
	" 24	30	0.142	4.26	156	39.8	12.3	Chloroform (2 cc.) in starch 2 hrs. after collection began.
	" 25	14	0.042	0.59	22	4.6	12.0	
	" 26	18	0.097	1.74	64	12.7	12.0	
	" 27	22	0.127	2.79	102	9.6	12.0	No clinical abnormali- ties.
	" 28	13	0.211	5.48	201	9.0		3 hr. collection.

on Monday, the preceding day is always one of a liberal mixed diet. These bile fistula dogs are usually very fond of meat and this is abundant in the usual Sunday feeding. This abundant meat feeding is known to cause a high bile acid output. A change to a carbohydrate diet reduces this level to the expected carbohydrate level almost within 24 hours. It is to be noted that the first experiment was started with a 3 day fasting period which would make the liver more vulnerable to the chloroform injury.

TABLE II.

Chloroform Anesthesia. Bile Salt Secretion Diminished.

Dog. No.	Date.	Vol. ume.	Amino nitrogen.		Tauro- cholic acid in 6 hrs.	Bili- rubin in 6 hrs.	Weight.	Remarks.
			In 1 cc. bile.	In 6 hrs.				
		cc.	mg.	mg.	mg.	mg.	lbs.	
17-34	Feb. 17	33	0.464	15.31	563	39.1	27.8	Fast began Feb. 15.
	" 18	20	0.309	6.18	223	34.8	27.3	Chloroform anesthesia (1 hr.) before col- lection.
	" 19	28	0.058	1.62	60	12.2	26.4	Carbohydrate diet.
	" 20	40	0.212	8.48	313	29.2	27.0	
	" 21	46	0.291	13.38	492	60.0	27.3	
	Mar. 31	47	0.342	16.0	587		28.5	Carbohydrate diet.
	Apr. 1	30	0.412	12.36	453	31.3	28.4	Chloroform anesthesia ($\frac{1}{2}$ hr.) after collec- tion.
	" 2	26	0.156	4.05	145	41.6	24.6	
	" 3	52	0.158	8.20	302	45.2	25.1	
	" 4	45	0.210	9.45	347	30.6	25.1	
	" 5	36	0.451	16.23	595	62.6	25.0	3 hr. collection.

TABLE III.

Chloroform Anesthesia.

Dog. No.	Date.	Vol. ume.	Amino nitrogen.		Tauro- cholic acid in 6 hrs.	Bili- rubin in 6 hrs.	Weight.	Remarks.
			In 1 cc. bile.	In 6 hrs.				
		cc.	mg.	mg.	mg.	mg.	lbs.	
19-3	Mar. 24	41	0.187	7.68	282	23.7	23.0	Carbohydrate diet.
	" 25	20	0.368	7.36	270	17.4	22.9	Chloroform anesthesia ($\frac{1}{2}$ hr.) after collec- tion.
	" 26	25	0.171	4.27	157	19.4	23.0	
	" 27	23	0.156	3.59	132	16.0	23.3	
	" 28	50	0.028	1.40	51	9.6	22.9	No clinical abnormali- ties.
	" 29	21	0.128	5.36	196	27.8	22.8	3 hr. collection.

Table III gives an interesting example of a reaction to chloroform anesthesia. The reaction on the 2 days following the anesthesia is quite moderate but definite. On the 3rd day we note a low bile acid output (about 15 per cent of normal) and a slight increase in total volume, making a great decrease in the concentration of bile acid per 1 cc. The bile pigments run a somewhat similar course, but the changes are much less marked. There was

TABLE IV.
Chloroform Anesthesia and Chloroform by Mouth.

Dog No.	Date.	Volume.	Amino nitrogen.		Taurocholic acid in 6 hrs.	Bilirubin in 6 hrs.	Weight.	Remarks.
			In 1 cc. bile.	In 6 hrs.				
21-45	Apr. 6	17	0.326	5.54	203	15.5	12.5	Carbohydrate.
	" 7	30	0.183	5.49	202	20.2	12.5	Chloroform anesthesia ($\frac{1}{4}$ hr.) before collection.
	" 8	24	0.169	4.05	149	29.3	13.0	No clinical abnormalities.
	" 9	10	Tr.	Tr.	Tr.	19.1	13.0	3 hr. collection.
	May 31	32	0.226	7.23	265	13.9	12.3	Carbohydrate diet.
	June 1	21	0.264	5.55	200	26.0	12.0	Chloroform (2.5 cc.) by mouth 3 hrs. after collection began.
	" 2		0.028			0.644	11.5	Part of collection lost in manipulation. Pigment estimated in 1 cc.
	" 3	28	0.126	3.52	129	21.4	11.5	
	Dog killed. Autopsy negative for chloroform injury. See below.							

no evidence of clinical abnormality at any time. This curious, delayed reaction is not common in this type of chloroform injury or disturbance.

Table IV gives an interesting contrast in the effect of chloroform by mouth and by anesthesia. Both experiments were done under uniform conditions on the same bile fistula dog with about 8 weeks intervening. This dog was surprisingly sensitive to chloroform anesthesia and $\frac{1}{4}$ hour gives a very striking change in bile

salt output. On the 2nd day the taurocholic acid falls practically to zero, while the total volume and bile pigment output remain almost unchanged. No clinical disturbance accompanied either of these experiments. When the chloroform is given by mouth we observe a similar reaction, but the fall in bile acid output comes the day after the chloroform by mouth, contrasting with a similar reaction on the 2nd day following chloroform anesthesia. This dog was killed with ether and autopsied carefully to make sure that no liver lesion could be disclosed which might explain these remarkable reactions. The autopsy protocol shows no histological basis for the reaction.

Autopsy.—Dog 21-45. Tan, mongrel female. (See Table IV.)

Dog killed by anesthesia 48 hours after administration of chloroform by mouth. The dog is in fair condition, but appears unusually thin on account of the loss of subcutaneous fat. Autopsy done at once. Thorax, heart, and lungs normal. Spleen very small, quite firm; cuts with difficulty.

Liver normal in size but is unusually dark yellowish green in color. The lobulation is easily made out. No evidence of necrosis. The bile ducts are slightly distended with bile. The gall bladder is well attached to the abdominal wall at the site of the operative fistula. The cystic and common ducts are slightly dilated. At the site of the operative excision the ligated common duct is about 0.8 cm. in diameter and presents a blind sac to the probe. About 3 cm. nearer the duodenum the continuance of the former duct may be made out shortly before it enters the papilla of Vater. The stomach, intestines, and other viscera are negative.

The bones are compared with those of a normal young dog killed at this time, sick with distemper. Ribs (Dog 21-45) show definite thinning of the trabeculae and shaft and the marrow is bright red. No spontaneous fractures. Femora (Dog 21-45) are thinned—shaft is about two-thirds as thick as normal control. Marrow is brick-red and cellular.

Microscopical sections made from all organs are of no significance for these experiments except those of the liver. Sections taken from different lobes of the liver show no necrosis and no fatty change in the liver cells. The marginal or portal hepatic epithelium is rich in glycogen and the central cells show a slight atrophy and pigmentation with fine yellow granules.

Table V shows the effects of two doses of phosphorus given subcutaneously. The first dose caused no change in volume output of bile, but there was an increase in the bile salt secretion. This may be a stimulus due to a small dose of this liver poison, but the mixed diet is a factor to be considered. This diet of

hospital table scraps was of variable make-up and often contained large amounts of meat. On such days the output of bile acids invariable increased. This experiment was done before this source of error was appreciated.

The second dose of phosphorus was not expected to be lethal, but there may have been a slight summation due to the dose of the preceding week. We note a gradual falling off in bile salt values especially marked in the 2 days before death. Even in this fatal

TABLE V.
Phosphorus Poisoning—Small and Large Doses.

Bile fistula and splenectomy.

Dog No.	Date.	Volume.	Amino nitrogen.		Taurocholic acid in 6 hrs.	Weight.	Remarks.
			In 1 cc. bile.	In 6 hrs.			
		cc.	mg.	mg.	mg.	lbs.	
16-41	Mar. 26	83	0.174	14.5	532	28.5	Mixed diet.
	" 27	66	0.240	15.9	582	28.5	10 mg. phosphorus in 2 cc. olive oil, intramuscularly.
	" 28	70	0.291	20.4	749	29.0	
	" 29	72	0.229	16.5	604	28.0	
	" 30	87	0.248	21.6	793	29.3	
	" 31	70	0.557	39.0	1,432	29.0	
	Apr. 2	63	0.539	34.0	1,248	29.0	Mixed diet.
	" 3	47	0.436	20.5	753	28.0	20 mg. phosphorus in 4 cc. olive oil, intramuscularly.
	" 4	41	0.595	24.4	896	27.5	
	" 5	23	0.760	17.5	643	27.5	
	" 6	40	0.227	9.1	334	27.3	
	" 7	38	0.200	7.6	279	27.0	
	" 8	Found dead in cage and autopsied immediately.					

case the influence on the bile salt secretion is slight as compared with the non-lethal chloroform influence. There was also a fall in bile volume secretion. The autopsy showed a definite liver pathology which appeared scarcely sufficient to cause death to a normal dog. This is in marked contrast to the absence of any histological findings in the chloroform dogs. Note the fall in the weight curve which is a good index of the clinical injury in this fatal phosphorus case.

Autopsy.—Dog 16-41. (See Table V.)

Thorax negative. Spleen absent, no accessory nodules. Marrow is deep red and cellular. Pancreas normal. Stomach and intestinal mucosa slightly engorged. Moderate cystitis of urinary bladder. Left kidney shows two pelvic stones. Parenchyma of kidneys normal.

Liver. Color normal, lobules conspicuous, no hemorrhages. Centers of lobules are pigmented, the edges are swollen and grey, but not necrotic. Bile ducts clean and slightly thickened. Common duct torn away from duodenum and fixed in old adhesions below liver; no bile in duodenum.

Microscopical sections. We are not concerned with the findings other than in liver sections. The liver sections were taken from different lobes. There are scattered liver necroses involving clusters of 2 to 4 liver cells. There is a healing reaction about such foci. There are numerous polymorphonuclear cells and phagocytes in all parts of the liver lobule. There is considerable fatty change involving the liver cells. Many liver cells appear quite normal and the liver does not seem to be fatally injured. This injury must have been minimal for the fatal outcome and perhaps would have been tolerated by a healthy, normal dog.

Table VI gives the results of two experiments with large doses of phosphorus given in oil hypodermically. The first experiment shows little change in bile salt output until the 3rd day after the injection when we note a fall to about 25 per cent of normal. There is a notable decrease in bile volume and throughout the experiment the concentration of bile salt per 1 cc. is high. If anything is to be said about the bile pigments it is to the effect that there may have been slight stimulus on the 2 days after the injection. Recovery was rapid after the end of the experiment. This dog was a "partial bile fistula" in which there was a small opening from the common duct into the duodenum, permitting bile to flow in small amounts into the duodenum during non-collection periods. This type of dog remains in perfect condition and is a valuable control on the "complete bile fistula dog" in which all bile is excluded from the duodenum.

The second experiment deals with a still larger dose of phosphorus which was expected to be lethal. As a matter of fact the dog was recovering when killed to examine the liver which showed evidence of only moderate injury. The initial reaction to this large dose of phosphorus (April 15) was most interesting, the bile salt content falling to a mere trace as in the chloroform experiments. There was a considerable recovery on the next 2 days,

but with a very low reading of bile salt on the day the dog was killed. This is the only experiment which gives us positive data on this initial reaction to phosphorus. The other injections were

TABLE VI.
Phosphorus Poisoning—Large Dose.

Dog. No.	Date.	Vol- ume.	Amino nitrogen.		Tauro- cholic acid in 6 hrs.	Bili- rubin in 6 hrs.	Weight.	Remarks.
			In 1 cc bile.	In 6 hrs.				
Partial bile fistula.								
18-30	Apr. 4	34	cc.	mg.	mg.	mg.	lbs.	Carbohydrate diet.
	" 5	12	0.384	13.05	479	22.2	31.0	
	" 6	12	1.460	17.52	643	37.6	29.0	
	" 7	12	0.789	9.46	347	36.6	28.5	20 mg. phosphorus at 11.00 a.m. in middle of collec- tion period.
	" 8	9	0.691	8.29	305	70.0	28.5	
	" 9	4	1.110	9.99	367	62.6	28.0	
	" 9	4	0.640	2.56	94	38.3	28.0	Recovery rapid.
Complete bile fistula.								
21-104	Apr. 13	68	0.182	12.37	454		35.5	Carbohydrate diet.
	" 14	50	0.070	3.50	129		35.0	
	" 15(a)	6 2	0.070	3.43	126		34.5	
	" 15(b)	40	Tr.	Tr.	Tr.			$\frac{3}{4}$ hr. collection fol- lowed by 35 mg. phosphorus, in- tramuscularly. 6 hr. collection after injection.
	" 16	44	0.042	1.84	68		34.0	
	" 17	40	0.042	1.68	62		33.0	
	" 18	35	0.028	0.98	36		33.0	

Dog not much intoxicated, ready to eat, lively. Killed with ether.

usually given in smaller doses during or after a collection. Clinically and histologically the recovery from a phosphorus injury of the liver is rapid.

Table VII gives the data for two experiments with medium sized doses of phosphorus. The first experiment shows a fall in

bile acid output on the day after the injection, but no change in volume and slight change in pigment. The second experiment with a slightly larger dose per kilo shows practically no change in the bile salt output. We are dealing here with one-half to two-thirds of a lethal dose of phosphorus which causes very little disturbance of the biliary secretion. A similar dose of chloroform would invariably cause a profound disturbance in the elimination of bile salts.

TABLE VII.
Phosphorus Poisoning.

Dog No.	Date.	Volume.	Amino nitrogen.		Taurocholic acid in 6 hrs.	Bilirubin in 6 hrs.	Weight.	Remarks.
			In 1 cc. bile.	In 6 hrs.				
		cc.	mg.	mg.	mg.	mg.	lbs.	
21-60	Feb. 8	30	0.296	8.88	326	17.6	17.0	Carbohydrate diet.
	" 9	21	0.155	3.24	119	14.8	16.0	
	" 10						16.0	8 mg. phosphorus after collection, intramuscularly.
	" 11	18	0.099	1.79	66	11.1	15.5	
	" 12	22	0.244	8.05	266	20.4	15.5	4 hr. collection.
	Apr. 4	18	0.184	3.31	122	5.8	18.0	Carbohydrate diet.
	" 5	37	0.170	6.29	231	20.0	16.5	
	" 6	17	0.241	4.09	150	12.2	16.5	12 mg. phosphorus in middle of collection period.
	" 7	30	0.155	4.05	170	18.6	16.5	
	" 8	20	0.225	4.50	166	10.9	16.3	
	" 9	9	0.199	3.58	122	22.3	16.3	3 hr. collection.

Table VIII gives us two more experiments with phosphorus given hypodermically. The first experiment deals with a dose of phosphorus about one-half lethal in amount. The most notable depression of bile salt excretion is on the day following the injection, but there are low values all through the after injection period. The pigment value and total volume are little influenced except total volume on the day after injection.

The second experiment may give some evidence for a stimulation of liver secretion of bile salt. The 2 days following the injection

show high values considerably above the control level. On mixed diet, however, one cannot be sure that the change is due to phosphorus—compare Table V. This dose of phosphorus was about one-third lethal and caused no untoward result.

TABLE VIII.
Phosphorus Poisoning.

Dog. No.	Date.	Volume.	Amino nitrogen.		Taurocholic acid in 6 hrs.	Bilirubin in 6 hrs.	Weight.	Remarks.
			In 1 cc. bile.	In 6 hrs.				
		cc.	mg.	mg.	mg.	mg.	lbs.	
19-3	Mar. 31	32	0.156	5.00	184	16.0	23.5	Carbohydrate diet. 14 mg. phosphorus before collection, intramuscularly.
	Apr. 1	39	0.057	2.22	82	16.6	23.3	
	" 2	11	0.100	1.10	40	11.5	23.0	
	" 3	36	0.072	2.60	95	15.7	22.8	
	" 4	32	0.084	2.69	99	16.7	22.5	
	" 5	11	0.113	2.48	91		22.5	3 hr. collection.
16-175	Mar. 26	80	0.170	13.6	499		22.0	Mixed diet.
	" 27	62	0.224	13.9	510		22.5	7.5 mg. phosphorus in 1.5 cc. olive oil, intramuscularly.
	" 28	54	0.306	16.6	610		23.0	
	" 29	82	0.207	16.9	621		23.2	
	" 30	39	0.220	8.6	316		24.0	
	" 31	58	0.175	10.2	375		23.3	

DISCUSSION.

Both chloroform and phosphorus are familiar liver poisons. The pathological effect of these poisons upon bile fistula dogs is very different. We note that a very small dose of chloroform by stomach or a short chloroform anesthesia may reduce the bile salt concentration of the bile to 10 per cent of normal or even less. A very small dose of phosphorus may have no effect or we may even suspect a stimulating action. Only when the dose of phosphorus is one-half or three-fourths of lethal do we note changes in the bile salt output. Even in lethal phosphorus poisoning we rarely observe the unusually low values for bile salt in the bile noted with mild chloroform injury.

How may we explain this remarkable difference? We know that these two poisons act very differently when we give amounts sufficient to cause recognizable liver cell injury. Chloroform causes a uniform, extensive, coagulative, hyaline necrosis with destruction of both nucleus and protoplasm of the cell. Phosphorus in lethal doses causes little hyaline necrosis, occasional clusters of cells appearing as hyaline masses, but extensive injury to the protoplasm of the liver cell which is full of fat droplets. In our experiments with phosphorus poisoning we can recognize some histological changes in the liver cell when we record definite fall in concentration of the bile salts. But in our chloroform experiments we cannot find any histological changes in the liver lobule referable to chloroform where we have observed these remarkable changes in bile salt concentration.

We must recall that in every chloroform experiment but one the dogs were on a liberal carbohydrate diet. This diet in itself will give protection against mild chloroform poisoning, and practically guarantee against liver injury recognizable by present histological methods (7, 1). Yet we must admit that the evidence for liver injury by the physiological test is conclusive.

It is believed by many that phosphorus acts chiefly upon cell protoplasm, and chloroform by contrast, chiefly upon the cell nucleus. It is, therefore, a possibility that the nucleus controls the bile salt metabolism of the liver cell and is much more sensitive to chloroform than to phosphorus. This observation illustrates again the important fact that function may be seriously disturbed in a gland cell without any lesions demonstrable by modern technique,—the functional test is better than the histological.

We are familiar with the fact that certain drugs may be stimulating in small doses and very toxic or lethal in large doses. We hoped to demonstrate a stimulating action by small doses of chloroform and phosphorus. It is probable that a dose of exactly the right amount might act as a stimulus to the formation of bile salts. All the doses of chloroform tried were depressant, but we are not convinced that a suitable dose might not give an overproduction of bile salts. We have two suggestive experiments with phosphorus, indicating a possible stimulus (Tables V and VIII). Both these experiments, unfortunately, were not on a uniform standard diet and it is possible that the increase observed was due to increase in proteins in the mixed diet.

SUMMARY.

Chloroform in remarkably small doses, incapable of causing recognizable histological liver injury or clinical reaction, can effect a profound decrease in bile salt concentration in fistula bile.

This action of these *small doses* of chloroform—a poison specific for the hepatic epithelium—gives more evidence that the bile salts are produced by the liver cell and not elsewhere in the body.

As chloroform acts strongly upon the cell nucleus it is suggested that the bile salt activity of the liver cell may be largely under the control of its nucleus.

Phosphorus in small doses may have no effect on the bile salt output or it may even have a stimulating influence. In large doses phosphorus does have a depressant effect upon bile salt output in fistula bile.

The bile volume may be slightly or not at all disturbed by small or moderate doses of these liver poisons.

The bile pigment at times shows a decrease or again is uninfluenced during these same periods.

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BILE SALT METABOLISM.

II. PROTEOSE AND X-RAY INTOXICATION. THYROID AND THYROXIN.

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These conditions may seem wholly unrelated yet they do have at least one thing in common—a disturbance or modification of endogenous nitrogen metabolism. Experiments in this laboratory (3) have shown that there is a distinct relationship between bile salt metabolism and normal endogenous nitrogen metabolism. For example, there is a definite level of bile salt production in fistula dogs on fasting. This bile salt production under such conditions is distinctly lowered by sugar feeding just as is noted in total urinary nitrogen. It is obvious that bile acids can be found from body cell protein and any protection of this protein breakdown (sugar feeding) is reflected in a decrease in bile salt output. Because of this fact we wished data on general conditions in which an increased breakdown of body protein was known to exist.

Proteose intoxication is a well known condition in which there is a severe initial shock, low blood pressure, vomiting and usually rapid recovery in a few days if a sublethal dose is given. There are many differences of opinion as to the chemical nature of the various substances used and their mode of action, but one thing is certain, there is a great rise in urinary nitrogen and blood non-protein nitrogen, indicating a considerable injury and breakdown of body protein. Whether the nucleus alone or cell protoplasm alone or both are injured is not known. The proteose used in these experiments was prepared from material obtained from closed intestinal loops by a method previously described (1).

We may safely assume in these experiments a very considerable increase in urinary nitrogen and consequent body protein catabolism following proteose intoxication. Does this cause a *rise* in bile salt excretion in fistula dogs? The tables below show a uniform *fall in bile salt output* following proteose injection. We may choose to explain this reaction on the basis of actual cell injury done the hepatic epithelium which is believed to produce the bile salt. There is no histological evidence of this injury, but very frequently we observe disturbance of function without histological change.

We believe that these experiments indicate strongly that the liver epithelium is seriously injured by this proteose. Whether other body cells are likewise injured is not clear except in the case of the kidney. McQuarrie and Whipple (1) showed that this proteose gives a temporary paralysis or impairment of function of the secretory cells of the kidney. There is no histological change and there is a return to normal kidney function within a few days following recovery. The change in the liver may be analogous, but the liver appears to be less sensitive to this poison than is the kidney.

The *x-ray experiments* are of equal interest, but the liver is not as sensitive to this agency as to proteose. Doses of x-ray given over the liver region have no influence in small doses up to about 30 per cent of the minimum lethal dose. When doses of 200 milliamperes minutes are given we may note a depression of the bile salt output which may be prompt or even delayed to the 2nd day. This decrease in bile salt output in no experiment reaches the low levels seen in chloroform, phosphorus, or proteose poisoning.

Experiments published from this laboratory (2, 5) give the necessary data to prove that the urinary nitrogen is increased under the above condition. We have likewise studied with care the histology of cases fatally poisoned by long x-ray exposures and have never found any histological evidence of liver epithelium injury. We have no fatal x-ray exposures in this bile fistula series, but the evidence speaks for a moderate grade of liver cell injury caused by the x-ray. This x-ray liver injury, of course, is relatively trifling compared with the destructive injury done certain body cells,—lymphocytes, germinal cells, and the mucosa cells of the small intestine.

In the case of the x-ray and proteose poisoning we note a fall in bile acid output associated with increase in urinary nitrogen due to body cell injury. We may choose to believe that the bile acids would increase but for the fact that the liver epithelium is actually injured by these agencies. We may believe that the parent substances which would normally go to the formation of taurocholic acid are not used by the liver cell and probably escape by way of the urine and contribute to the excess of urinary nitrogen which is so constant under these experimental conditions. But it may be that these intoxications are associated with a profound disturbance of the whole endogenous nitrogen metabolism which completely upsets the normal cycle. It is not surprising that the abnormal cycles are obscure when we are in almost complete ignorance as to the normal endogenous bile salt metabolism.

Thyroid extract by mouth or *thyroxin* subcutaneously will speed up and perhaps modify the endogenous protein metabolism. We know from other experiments that thyroid in these doses in dogs will cause a definite rise in urinary nitrogen. Whatever the reaction may be within the animal body we cannot record any recognizable change in the bile salt output. The bile salt production evidently is not controlled by the thyroid nor modified by the active principle of this gland.

Method.

The general routine of bile collection, animal care, etc., are reviewed in the first paper of this series. The proteose used was obtained from closed loops of the small intestine in dogs by a method described elsewhere (1). The x-ray technique has been described elsewhere (6) and consists of exposures to the x-ray given over the liver area. Spark-gap 10.5 inches—95 kilovolts—2 mm. aluminum filter and body target distance of 10 inches. Other parts of the body were screened by 1 mm. sheet lead. Animals were given morphia to insure quiet during the long x-ray exposures. The exercise or control experiments deal with short, brisk exercise periods, usually a run on leash for the period stated.

EXPERIMENTAL OBSERVATIONS.

Experiments with proteose intoxication in bile fistula dogs are difficult. If the intoxication is severe the dog is so prostrated that

it is difficult to obtain a bile collection. With a lethal dose the intoxication may be so acute that there is not sufficient time for a collection before death. From a number of experiments we submit three given below, realizing that they are incomplete in many respects. The evidence for injury of the hepatic epithelium is beyond question. In all cases there is a moderate increase in bile volume as is usually noted after proteose intoxication. The

TABLE IX.
Proteose Intoxication—Severe and Lethal Doses.

Dog No	Date	Volume	Amino nitrogen		Taurocholic acid in 6 hrs	Weight	Remarks
			In 1 cc bile	In 6 hrs			
		cc	mg	mg	mg	lbs	
15-22	Sept. 10	35	0.878	30.7	1,134	35.5	Mixed diet.
	" 11	21	1.09	22.9	841	33.8	
	Sept. 12	½ lethal dose of proteose. Severe reaction in 5 min.					
18 30	Sept 12	44	0.351	15.45	567	34.5	Very weak and sick. Improving.
	" 13	22	0.197	4.33	159		
	" 16	48	0.30	14.33	526	31.5	
	June 10	8	0.634	5.07	186	27.4	Carbohydrate diet.
	" 11	9	0.633	5.69	209	27.1	
	" 13	9	2.168	19.51	716	28.0	
	June 14	112 cc. proteose before starting collection. Profound effect began immediately.					
June 14	21	0.322	6.76	248	27.8		
" 15	12 m. Death.						

bile salt level may be immediately reduced, but usually not until the day after the injection,—in one experiment (Table X) to a mere trace following an extreme grade of intoxication.

Table IX gives the data on two experiments with proteose intoxication, one severe and the other lethal. The first experiment shows a severe reaction with an apparent depression of bile salt output on the day of the injection. There is no question about this decrease on the following day when the bile salt level

is scarcely 20 per cent of the normal. There is a slow improvement in the next few days and a return of the bile salt level toward normal. The second experiment shows a lethal reaction. There is little or no decrease of bile acid output during the collection immediately following the injection and severe initial shock. The following morning, unfortunately, no sample of bile was obtained for analysis.

Table X shows a lethal proteose experiment in which there is some evidence for an actual *stimulus* of bile salt excretion immediately following the injection and initial shock. There was a pro-

TABLE X.
Lethal Proteose Intoxication.

Dog. No.	Date.	Volume	Amino nitrogen.		Tauro- cholic acid in 6 hrs	Weight	Remarks.
			In 1 cc bile	In 6 hrs.			
		cc.	mg.	mg.	mg.	lbs.	
21-103	June 13	36	0 54	1 94	712	30	Carbohydrate diet.
	" 14	16	0 07	1 12	41	28	
	June 15	230 M.A.M. x-ray before collection.					
	June 15	30	0 14	4 20	154	28	
	" 16	26	0 196	5 09	187	27	
	June 17	Minimum lethal proteose injection. Characteristic reaction at once.					
	June 17	37	0 209	7 71	284	27	Killed with ether.
" 18	20	0	0	None	26		

found drop in bile salt output on the following day with values which could not be measured by our methods. This dog, killed with ether after this period of collection, showed a liver which was practically normal in gross and in histological sections. The x-ray experiment in this table is discussed below.

Table XI gives the data on two x-ray experiments of different dosage. The first dog received 100 milliamperes minutes and showed no clinical symptoms. The level of bile salt output shows no significant change related to this exposure. The second dog received 200 milliamperes minutes and showed no clinical symp-

TABLE XI.
X-Ray Exposure.

Dog No.	Date.	Volume.	Amino nitrogen.		Taurocholic acid in 6 hrs.	Pigment in 6 hrs.	Weight.	Remarks.
			In 1 cc. bile.	In 6 hrs.				
17-34	Mar. 24	45	0 388	17.46	639	38.3	29.0	Carbohydrate diet.
	" 25	32	0 311	14 93	548	38 4	28.8	
	Mar. 25	4 hr. collection followed by x-ray exposure of 100 M.A.M.						
	Mar. 26	50	0.372	18.6	682	69.6	28.1	No clinical symptoms. 4 hr. collection.
	" 27	48	0.242	11 6	435	66.8	28.0	
	" 29	34	0 399	20.34	745	40.8	27.8	
19-3	Mar. 10	60	0.172	10.30	377		22.4	Carbohydrate diet.
	" 11	18	0.294	7.94	291		22.0	
	Mar. 11	4 hr. collection followed by x-ray exposure of 200 M.A.M.						
	Mar. 12	34	0.127	4.32	159		23 6	No clinical symptoms. 4 hr. collection.
	" 13	38	0.058	2.2	81		22 1	
	" 14	38	0.116	4.41	162		23 2	
	" 15	40	0.118	7.08	260		23.2	

TABLE XII.
X-Ray Exposure. Chloroform by Mouth.

Dog No.	Date.	Volume.	Amino nitrogen.		Taurocholic acid in 6 hrs.	Weight.	Remarks.
			In 1 cc. bile.	In 6 hrs.			
		cc.	mg.	mg.	mg.	lbs.	
21-60	June 13	29	0.112	3.25	120	16.8	Carbohydrate diet.
	June 14	Collection preceded by 230 M.A.M. x-ray.					
	June 14	20	0.098	1.96	72	16.0	1 hr. collection.
	" 15	26	0.126	3.28	120	15 0	
	" 16(a)	8	0.314			14.8	
	June 16	2 cc. chloroform by mouth.					
	June 16 (b)	24	0.126	3.02	111		Vomiting and acutely ill.
	" 17	5	Tr.	Tr.	Tr.	15.0	
" 18	< 1	0	0	0	14.0	Killed with ether.	

TABLE XIII.
Thyroid and Thyroxin Feeding.

Dog. No.	Date.	Volume.	Amino nitrogen.		Tauro- cholic acid in 6 hrs.	Pig- ment in 6 hrs.	Weight.	Remarks.
			In 1 cc. of bile.	In 6 hrs.				
		cc.	mg.	mg.	mg.	mg.	lbs.	
19-39	Sept. 20	34	0.239	8.13	299	35.5	28.5	Carbohydrate diet.
	" 21	42	0.211	8.86	325	27.74	28.0	2 gm. thyroid in p.m.
	" 22	42	0.199	8.36	307	32.12	28.0	1 " " before and after collection.
	" 23	41	0.198	8.12	298	29.24	27.5	2 gm. thyroid before col- lection.
	" 24	34	0.199	6.76	248	35.5	27.8	
	" 25	15	0.213	6.40	235	31.32	28.3	3 hr. collection.
18-30	Sept. 20	24	0.914	21.95	806	36.8	39.0	Carbohydrate diet.
	" 21	40	0.382	15.30	562	36.16	38.8	2 gm. thyroid after col- lection.
	" 22	45	0.595	26.75	982	42.84	39.0	1 gm. thyroid before and after collection.
	" 23	40	0.313	12.50	459	33.40	38.0	2 gm. thyroid before col- lection.
	" 24	20	0.924	16.50	680	34.8	38.0	
	" 25	12	0.881	21.20	780	33.44	38.5	3 hr. collection
18-30	May 24	53	0.358	18.97	697		35.5	Carbohydrate diet.
	" 25	20	0.884	17.68	649		34.8	
	" 26	28	0.442	12.37	454			16 mg. thyroxin (44 ⁷ / ₁₀₀ per cent) in food.
	" 27	27	0.489	13.20	485			16 " " subcu- taneously.
	" 28	21	0.614	12.89	473			
	" 29	15	0.35	10.50	387			3 hr. collection.
21-54	Jan. 11	36	0.281	10.11	371		22.5	Carbohydrate diet.
	" 13	21	0.325	6.82	250		22.5	
	" 14	14	0.661	9.25	340		20.5	12 mg. thyroxin (pure) subcutaneously.
	" 15	21	0.489	10.27	377			
	Jan. 17	30	0.807	24.21	889	47.7	23.0	Mixed diet.

Thyroxin obtained through the generous interest of Dr. E. C. Kendall of the Mayo Clinic.

TABLE XIV.
Exercise—Control.

Dog No.	Date.	Volume.	Amino nitrogen.		Taurocholic acid in 6 hrs.	Weight.	Remarks.
			In 1 cc. bile.	In 6 hrs.			
		cc.	mg.	mg.	mg.	lbs.	
21-60	May 31	30	0.139	4.17	153	16.5	Carbohydrate diet.
	June 1	29	0.18	5.22	192	16.0	Exercise $\frac{3}{4}$ hr.
	" 2	32	0.113	3.62	133	15.5	" 1 hr.
	" 3	14	0.294	4.12	151	15.5	
	" 4	5	0.30	3.00	110	15.5	3 hr. collection.
21-60	May 23	42	0.21	8.82	324	16.3	Carbohydrate diet.
	" 24	36	0.17	6.12	224	16.0	
	" 25	31	0.196	6.08	223	16.0	Exercise $\frac{3}{4}$ hr. before collection.
	" 26	40	0.097	3.88	142	16.5	Exercise 1 hr. before collection.
	" 27	26	0.226	5.87	215	16.0	
	" 28	9	0.211	3.80	148		3 hr. collection.
21-103	May 31	68	0.222	15.09	554	30.3	Carbohydrate diet.
	June 1	63	0.18	11.34	416	28.5	Exercise $\frac{3}{4}$ hr. before collection.
	" 2	70	0.142	9.94	365	29.0	Exercise 1 hr. before collection.
	" 3	40	0.196	7.84	288	29.3	
	" 4	17	0.155	5.26	193	29.0	3 hr. collection.
21-104	May 16	37	0.042	1.55	57	36.3	Carbohydrate diet.
	" 17	55	0.085	4.67	171	36.0	
	" 18	62	0.042	2.60	95	36.0	Exercise $\frac{3}{4}$ hr. before set up.
	" 19	63	0.028	1.76	65	36.0	
	" 20	45	0.042	1.89	69	36.0	

Fistula drained $\frac{1}{2}$ hr. before exercise in all experiments and before collection on days following each exercise day.

toms, but a definite fall in the bile salt output on the 2 days following the x-ray exposure. This decrease was more marked in the second 24 hours and, subsequently, we note a return to normal on the 4th day.

Table XII is to be compared with the experiment in Table X in which the dog was also given 230 milliamperes minutes. Dog

21-103 (Table X) shows no disturbance in bile salt output, but Dog 21-60 (Table XII), with the same dose of x-ray, shows a slight initial depression on the day of the exposure. The next day the bile salt output is normal. The 2nd day a dose of chloroform is given and this confused the picture. It is not quite clear why a fatal reaction followed this dose of chloroform—perhaps a combination of the x-ray and chloroform effects gives the correct explanation.

Table XIII gives the data on two feeding experiments with thyroid tablets and two thyroxin experiments. All the data indicate that thyroid by mouth or thyroxin subcutaneously do not influence the bile salt output. We are confident that enough thyroid was given to elicit the usual response and cause an increase in urinary nitrogen.

Table XIV gives control data which is of value as a base line for the other experiments. These experiments show that periods of brisk exercise of $\frac{3}{4}$ to 1 hour's duration do not modify the excretion of bile salts in bile fistula dogs.

SUMMARY.

Proteose intoxication is associated with a fall in bile salt output in fistula dogs in spite of the well known increase in body protein catabolism. It is probable that this reaction is an indication of real injury done the liver cell by the proteose. In this respect proteose intoxication resembles phosphorus poisoning.

X-ray exposure over the liver (one-third minimum lethal dose) does not usually modify the bile salt output, but 200 milliamperes minutes or more given over the liver may be expected to cause some decrease in bile salt output. This indicates a slight or moderate injury of the hepatic epithelium by the x-rays even though lethal dosages give no evidence of histological injury of liver epithelium.

Thyroid extract and thyroxin given in sufficient dosages to cause an increase in the urinary nitrogen do not modify appreciably the bile salt output under the conditions of our experiments.

Bile salt output, following brisk *exercise* periods ($\frac{3}{4}$ to 1 hour), shows a normal level and serves as adequate controls for the above experiments.

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BILE SALT METABOLISM.

III. GELATIN, FISH, YEAST, COD LIVER, AND MEAT EXTRACTS.

BY FRANCIS S. SMYTH AND G. H. WHIPPLE.

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(Received for publication, February 18, 1924.)

The most suggestive observation of this series is to the effect that *gelatin* feeding causes no demonstrable increase in bile salt output. This is such an important point that more experiments should be performed to establish this observation beyond a doubt. Gelatin should be given with various other diet factors to observe its influence, if any, upon the bile salt output. We were unable to complete these important experiments and must publish this work as it stands (Table XV) with the hope of continuing this problem at an early date. If it is a fact that gelatin does not increase the bile salt output we have a hint that certain amino acids lacking in gelatin (*e.g.* tryptophane and tyrosine) may be concerned with the construction of cholic acid. Cholic acid is the delimiting factor in the output of bile acid as there always appears to be an excess of taurine in the animal body (2). We know almost nothing about cholic acid as to its origin and fate in the body.

Many tables in the two preceding papers give data to show the prompt rise in bile acid output which follows a change from a carbohydrate diet to a meat diet or to a mixed diet rich in meat. In general the more meat protein in the diet the more bile acids secreted in the bile. An earlier publication from this laboratory (1) established this point and in that paper the work of other investigators was reviewed. We have observed in experimental work on anemia that meat protein favors rapid regeneration of hemoglobin, but fish protein is inert. For this reason, particularly, we studied carefully the influence of a fish diet on these

bile fistula dogs. This type of fish protein (canned, high colored salmon) causes the same rise in bile salt output as is observed after meat feeding.

Many experiments were made with extracts of various kinds: water extracts of meat and liver, commercial meat extract (Liebig's), and alcoholic extracts of meat and liver. As far as our experience goes these extracts are all negative or inert when given by mouth to bile fistula dogs. Table XVII gives several experiments with commercial beef extract which are negative. It did not seem worth while to publish the experiments dealing with the other extracts.

EXPERIMENTAL OBSERVATIONS.

The technique of these experiments is exactly like that described in the first paper of this series. Practically all these food substances are readily eaten when given alone or with various standard diets. The gelatin was of good commercial grade dissolved in warm water, sometimes flavored with salt or meat extract and given by stomach tube just before the beginning of the regular collection.

Most of the dogs used in these experiments were "complete bile fistulas" by which we mean complete exclusion of bile from the duodenum as established by autopsy at some subsequent date. Dogs 18-30 and 15-22 are spoken of as "incomplete fistulas" by which we mean that a little bile could seep into the duodenum during resting periods because there was a tiny fistulous tract connecting the severed common duct and duodenum. This slight night drainage of bile into the intestine makes all the difference between perfect health in the incomplete fistula and the various ailments of the complete bile fistula which we have reviewed in various publications (3).

Table XV shows uniformly negative reaction to the feeding of gelatin except in the last experiment in the last dog. There seems to be a rise in bile acid output in this single experiment, but we feel that this should be discounted in the face of all other negative results. There seems to be no change in the bile pigment output. The carbohydrate feeding continued throughout all the week of these experiments, supplemented by the gelatin solution at the beginning of the collections as indicated in the tables.

Table XVI shows that salmon muscle has exactly the same effect on bile salt output as does skeletal beef muscle. The carbohydrate diet is replaced by the fish feeding as indicated in the table

TABLE XV.
Gelatin Feeding.

Dog No.	Date.	Volume.	Amino nitrogen.		Taurocholic acid in 6 hrs.	Pigment in 6 hrs.	Weight.	Remarks.
			In 1 cc. bile.	In 6 hrs.				
		cc.	mg.	mg.	mg.	mg.	lbs.	
15-22	Apr. 26	12	0.672	8.07	296	41.8	31.3	Carbohydrate diet.
	" 27	35	0.452	15.83	581	38.8	32.5	" "
	" 28	23	0.474	10.90	400	84.0	31.5	30 gm. gelatin with beef extract.
	" 29	17	0.61	10.37	381	71.1	30.5	50 gm. gelatin.
	" 30	17	0.605	10.28	377	47.3	30.1	Carbohydrate diet.
18-30	Apr. 26	17	0.686	11.66	428	53.2	34.3	Carbohydrate diet.
	" 27	20	0.747	14.94	549	55.6	34.3	" "
	" 28	18	0.78	14.04	516	81.4	34.0	30 gm. gelatin, a.m. and p.m.
	" 29	8	0.936	7.49	272	40.4	33.3	50 gm. gelatin a.m.
	" 30	12	0.676	8.11	298	47.5	34.0	Carbohydrate diet.
	May 1	10	0.506	10.12	372	41.8	34.0	3 hr. collection.
17-181	Sept. 3	61	0.198	12.00	440		25.5	Cracker and beef heart.
	" 4	41	0.166	6.72	247		24.5	" " " "
	" 5	13.5	0.395	5.33	196		24.5	100 gm. gelatin before set up.
	" 6	29	0.331	9.60	353		24.8	100 gm. gelatin before set up.
	Sept. 10	49	0.292	14.3	525		25.0	Mixed diet.

on 2 days of each week. The increase in bile volume always noted after meat feeding is likewise present after fish feeding, but probably to a less extent than would have been observed with like amounts of cooked beef.

We record peculiar observations with yeast and cod liver feeding. In some experiments there seems to be an actual inhibition of bile salt excretion after feeding yeast or cod liver. Two experiments in each group show a suggestive inhibition and the other experiment

TABLE XVI.
Salmon Feeding.

Dog No.	Date.	Volume.	Amino nitrogen.		Taurocholic acid in 6 hrs.	Weight.	Remarks.
			In 1 cc. bile.	In 6 hrs.			
		cc	mg.	mg.	mg.	lbs.	
21-45	Mar. 22	34	0.426	14.48	537	13.3	Carbohydrate diet.
	" 23	30	0.227	6.81	251	13	
	" 24	28	0.227	6.35	233	12.8	450 gm. salmon, a.m. and p.m.
	" 25	45	0.437	19.66	721	13.5	450 " " "
	" 26	18	0.326	11.72	430	13.3	3 hr. collection.
21-60	Mar. 7	34	0.185	6.29	231	16.3	Carbohydrate diet.
	" 8	29	0.143	4.15	153	16	
	" 9	39	0.273	10.65	392		450 gm. salmon, a.m. and p.m.
	" 10	42	0.451	18.94	685		450 " " "
	" 11	26	0.397	10.32	379	15.3	
	" 12	10	0.425	8.50	312	15.3	3 hr. collection.
15-22	Apr. 5	46	0.398	18.31	674	32.3	Carbohydrate diet.
	" 6	12	0.678	8.13	297	32	
	" 7	44	0.296	13.02	477	32	500 gm. salmon, a.m. and p.m.
	" 8	52	0.509	26.47	974	32	500 " " "
	" 9	28	0.269	7.53	276	32	
	" 10	4.2	0.955	8.02	294	32	3 hr. collection.

We are indebted to the Alaska Packing Corporation for this supply of canned salmon used in our experiments.

is negative. What the significance of this observation may be we cannot say. In the absence of more experiments to confirm this peculiar reaction it would be futile to discuss the interesting possibilities. It is obvious that larger doses of these food substances should be given alone and combined with various food factors.

Beef Extract Feeding.

Dog No.	Date.	Volume.	Amino nitrogen.		Taurocholic acid in 6 hrs.	Pigment in 6 hrs.	Weight.	Remarks.
			In 1 cc. bile.	In 6 hrs.				
		cc.	mg.	mg.	mg.	mg.	lbs.	
18-30	Oct. 6	57	1.405	80	2,937	30.3	40	Carbohydrate diet.
	" 7	38	0.468	17.60	646	39.7	38.8	
	" 8	45	0.298	13.40	492	47.4	39	15 gm. beef extract before set up.
	" 9	47	0.454	21.25	780	49.6	39	15 gm. beef extract before and after collection.
	" 10	35	0.468	16.40	602	48.7	38.8	
	" 11	15	0.798	24	881	34.4	39.3	3 hr. collection.
19-39	Oct. 6	45	0.211	9.50	349	40.2	30	Carbohydrate diet.
	" 7	45	0.298	13.40	492	35.2	29.8	
	" 8	45	0.156	7.03	258	29.1	29.9	15 gm. beef extract before and after collection.
	" 9	45	0.255	11.45	420	29.1	30	15 gm. beef extract before and after collection.
	" 10	40	0.241	9.04	354	29.2	30.3	
	" 11	17	0.280	9.52	350	33.2	29.5	3 hr. collection.
18-137	Oct. 7	30	0.185	5.55	204		22.5	Cracker and beef heart diet.
	" 8	35	0.188	6.38	234		23	
	" 9	34	0.170	5.76	211		22	15 gm. beef extract before collection.
	" 10	43	0.124	5.33	196		23	15 gm. beef extract before collection.
	" 11	40	0.168	6.72	246		24.3	15 gm. beef extract before collection + 1 gm. taurine.
	Oct. 14	48	0.213	10.2	370		24.3	Mixed diet.
17-181	Oct. 7	43	0.258	11.1	408		25	Cracker and beef heart diet.
	" 8	28	0.215	6.02	221		24.5	
	" 9	40	0.198	7.92	291		24.5	15 gm. beef extract before collection.
	" 10	40	0.165	6.60	242		24.5	15 gm. beef extract before collection.
	" 11	38	0.225	8.55	314		25	15 gm. beef extract before collection + 1 gm. taurine.
	Oct. 14	61	0.399	24.3	892		25.3	Mixed diet.

TABLE XVIII.
Yeast Feeding.

Dog No.	Date.	Volume.	Amino nitrogen.		Taurocholic acid in 6 hrs.	Weight.	Remarks.
			In 1 cc. bile.	In 6 hrs.			
		cc.	mg.	mg.	mg.	lbs.	
19-39	Oct. 27	53	0.226	12	440	29	Carbohydrate diet.
	" 28	45	0.184	8.30	305	29	20 gm. yeast after collection.
	" 29	40	0.141	5.64	207	28.5	15 " " before and after collection.
	" 30	45	0.115	5.18	190	28.3	30 gm. yeast before collection.
	" 31	40	0.169	6.76	248	28.5	"
	Nov. 1	17	0.239	8.12	297	28	3 hr. collection.
15-22	Oct. 27	37	0.306	11.20	412	34	Carbohydrate diet.
	" 28	72	0.139	10.00	367	34	20 gm. yeast after collection.
	" 29	37	0.212	7.85	289	33.3	15 " " before and after collection.
	" 30	23	0.244	5.62	206	33	30 gm. yeast before collection.
	" 31	65	0.295	19.15	704	34.5	
18-30	Oct. 27	23	0.696	16.00	585	39	Carbohydrate diet.
	" 28	40	0.706	28.25	1035	39	20 gm. yeast after collection.
	" 29	52	0.382	19.89	725	38.8	15 " " before and after collection.
	" 30	35	0.273	9.56	350	37.8	30 gm. yeast before collection.
	" 31	43	0.338	14.55	535	39	
	Nov. 1	20	0.295	11.80	433	39.3	3 hr. collection.

Compressed yeast in cakes (Fleischmann's) used.

TABLE XIX.
Cod Liver Feeding.

Dog No.	Date.	Volume.	Amino nitrogen		Taurocholic acid in 6 hrs.	Weight.	Remarks.
			In 1 cc. bile.	In 6 hrs.			
		cc.	mg.	mg.	mg.	lbs.	
21-103	Apr. 19	50	0.28	14	515	31	Carbohydrate diet.
	" 20	24	0.099	2.38	88	30.5	50 gm. dried cod liver.
	" 21	60	0.042	2.52	91	30	75 " " " "
	" 22	60	0.20	12 0	440	30	
	" 23	14	0.27	7.56	289	29	3 hr. collection.
21-60	Apr. 18	27	0.183	4.94	181	18	Carbohydrate diet.
	" 19	31	0.255	7.90	287	16.8	
	" 20	36	0.085	3.06	112	16.3	20 gm. dried cod liver.
	" 21	29	0.112	3.25	119	16.5	20 " " " "
	" 22	45	0.10	4.50	165	16	
	" 23	9	0.343	6.18	226	16.5	3 hr. collection.
21-45	Apr. 18	24	0.324	7.78	287	13.5	Carbohydrate diet.
	" 19	39	0.224	8.74	321	13	
	" 20	45	0.142	10.58	388	13.3	4 hr. collection.
	" 21	19	0.223	4.24	156	13.3	30 cc. cod liver oil.
	" 22	37	0.257	9.51	350	12.8	30 " " " "
	" 23	9	0.329	5.92	215	13	3 hr. collection.

Dried cod liver furnished through the courtesy of Dr. Langley Porter.

SUMMARY.

Gelatin feeding in contrast to meat feeding does not increase the bile salt output. If confirmed by subsequent experiments this may indicate that some amino acid lacking in gelatin (*e.g.* tryptophane) may be the precursor of cholic acid.

Salmon muscle like beef muscle feeding causes a marked increase in the bile salt output in fistula bile. This is in contrast to the influence upon hemoglobin formation in the body. Beef muscle stimulates hemoglobin production in anemia, but fish muscle is inert.

Commercial *beef extract* has no influence on bile salt output in fistula bile in our experiments. Likewise watery extracts of meat and liver as well as alcoholic extracts of meat and liver made in the laboratory were inert.

Yeast and cod liver feeding shows a suggestive inhibitory reaction which we cannot explain. Further experiments may give the necessary data for a complete understanding of this phenomenon.

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BILE SALT METABOLISM.

IV. NEGATIVE INFLUENCE OF DRUGS, ATROPINE, PILOCARPINE, PHLORHIZIN, QUININE, ETC.

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Much has been written at various times concerning the cholagogue action of various drugs. By some workers a cholagogue action was claimed for this or that drug, but the cholagogue effect was as often denied by other workers. This indicates that these drugs have no important cholagogue action such as is constantly observed after exhibition of whole bile or bile salts. In another place this subject has been recently reviewed (2).

All the drugs listed in this paper and many others have been said by various authors to influence bile flow or the bile solid output (usually bile salts indicated). It will be noted that all our tabulated experiments show negative results. We have performed other experiments under identical conditions dealing with *calomel*, *hydrochloric acid*, *soaps*, *sodium salicylate*, and *alcohol*. All this series of observations was likewise negative, but it did not seem necessary to publish this negative data.

EXPERIMENTAL OBSERVATIONS.

The technical procedure and experimental detail of these experiments were exactly like those described in the other papers of this series.

Table XX shows several experiments each with atropine and pilocarpine. All the experiments with subcutaneous injection of these two drugs are negative in bile fistula dogs. The data

TABLE XX.
Atropine and Pilocarpine.

Dog No.	Date.	Volume.	Amino nitrogen.		Taurocholic acid in 6 hrs.	Weight.	Remarks.
			In 1 cc. bile.	In 6 hrs.			
		cc.	mg.	mg.	mg.	lbs.	
21-60	Apr. 11	36	0.099	3.56	131	16.3	Carbohydrate diet.
	" 12	32	0.084	2.69	98	16.5	
	" 13	35	0.168	5.88	215	16.0	4 mg. atropine sulfate subcutaneously before set up.
	" 14	29	0.143	4.15	152	15.8	4 mg. atropine sulfate subcutaneously before set up.
	" 15	25	0.156	3.90	143	15.8	4 mg. atropine sulfate subcutaneously before set up.
21-45	Apr. 11	40	0.17	6.80	250	13.0	Carbohydrate diet.
	" 12	40	0.182	7.28	267	13.3	
	" 13	35	0.202	7.07	260	13.0	4 mg. atropine sulfate subcutaneously before collection.
	" 14	33	0.214	7.06	259	12.5	4 mg. atropine sulfate subcutaneously before collection.
	" 15	26	0.212	5.51	202	12.5	
21-45	Mar. 28	41	0.199	8.15	299	13.0	Carbohydrate diet.
	" 29	24	0.28	6.72	247	13.0	
	" 30	23	0.241	5.54	203	13.0	10 mg. pilocarpine subcutaneously before collection.
	" 31	32	0.336	10.75	395	12.5	
	Apr. 1	18	0.351	6.37	234	12.5	5 mg. pilocarpine subcutaneously before collection.
	" 2	5	0.35	3.50	128	12.5	3 hr. collection.
21-60	Mar. 29	45	0.098	4.41	162	16.3	Carbohydrate diet.
	" 30	35	0.199	6.96	255	17.3	10 mg. pilocarpine subcutaneously before collection.
	" 31	37	0.21	7.77	285	16.8	
	Apr. 1	30	0.212	6.36	234	16.3	5 gm. pilocarpine subcutaneously before collection.
	" 2	14	0.099	2.76	101	16.0	3 hr. collection.

seem conclusive that under the conditions of these experiments neither the bile volume nor bile salt output is influenced by a therapeutic dose of atropine or pilocarpine.

Phlorhizin is a drug much used in physiology. Certain points relative to its effect on body tissues and fate in the organism are in dispute, but there seems to be no doubt that this drug in the doses given causes a considerable mobilization of carbohydrate throughout the body. It is highly probable that the liver is

TABLE XXI.
Phlorhizin.

Dog No.	Date.	Volume.	Amino nitrogen.		Taurocholic acid in 6 hrs.	Weight.	Remarks.
			In 1 cc. bile.	In 6 hrs.			
		cc.	mg.	mg.	mg.	lbs.	
21-54	Jan. 24	41	0.14	5.74	211	22	Carbohydrate diet.
	" 25	56	0.195	10.95	402	22	
	" 26	36	0.167	6.01	221	23.3	
	" 27	48	0.173	8.30	305	22.5	
	" 28	47	0.255	11.98	440	22.3	0.5 gm. phlorhizin subcutaneously before collection.
	" 29	47	0.227	10.67	392	22.3	
16-41	Jan. 31	87	0.381	33.2	1,219	35.5	Mixed diet.
	Feb. 1	115	0.236	27.2	998	36	0.2 gm. phlorhizin subcutaneously before collection.
	" 2	92	0.361	33.3	1,222	35.5	1.0 gm. phlorhizin subcutaneously before collection.
	" 3	109	0.287	32.5	1,193	36	
	" 4	88	0.439	39.6	1,452	36.2	
	" 5	110	0.350	39.4	1,450	36.3	

involved in this carbohydrate mobilization. It will be seen from Table XXI that neither the bile volume nor the bile salt output is influenced by phlorhizin subcutaneously.

Some suggestive experiments by Siperstein and Litman (1) indicate that therapeutic doses of quinine may cause actual cell injury in the liver. Their experiments were done on rabbits. Our experiments in bile fistula dogs give no evidence of any serious injury done the liver by large therapeutic doses. Other experi-

ments in which quinine was given by mouth showed considerable clinical disturbance, but no notable change in bile salt output.

We have observed negative results with therapeutic doses of *calomel*, *sodium salicylate*, and *hydrochloric acid*. It has been suggested at various times that these substances do influence bile flow and bile solids.

Our experiments with ethyl alcohol were not perfectly satisfactory. In some experiments there is an apparent fall in bile and bile acid output and we cannot deny that doses of alcohol sufficient to produce somnolence and semicoma may

TABLE XXII.

Quinine.

Dog No.	Date.	Volume.	Amino nitrogen.		Taurocholic acid in 6 hrs.	Pigment in 6 hrs.	Weight.	Remarks.
			In 1 cc. bile.	In 6 hrs.				
21-104	May 3	55	0.127	6.98	256	80	38	Carbohydrate diet. 182 mg. quinine intravenously before set up.
	" 4	43	0.168	7.22	265	82	37	
	" 5	53	0.198	10.49	385	76	36	
	" 6	53	0.198	10.49	385	70	36	
18-30	May 2	12	0.527	6.32	231		31	Carbohydrate diet. 135 mg. quinine intravenously before set up.
	" 3	11	0.564	6.20	228		29	
	" 4	6	0.588	3.53	129		28	
	" 5	10	0.622	6.22	228		28.3	
	" 6	16	0.78	12.48	460		28.3	

reduce slightly the bile flow and bile salt output. But these experiments are difficult because of the fact that these intoxicated dogs are very difficult subjects for bile collection and it is hard to be quite certain that some bile did not escape during the period of excitement. The usual dose of ethyl alcohol by stomach causes a typical intoxication with a preliminary period of excitement during which the dog may try to stand on its head or climb all over the laboratory furniture. One or two experiments will convince a sceptic that it is difficult to make quantitative collections under these conditions of alcoholic euphoria.

SUMMARY.

Atropine and pilocarpine have no influence on bile salt output in bile fistula dogs under the conditions of these experiments. Phlorhizin and quinine are negative under similar conditions.

Calomel, hydrochloric acid, sodium salicylate, and alcohol in our experience have no constant influence upon output of bile salt in fistula bile.

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A STUDY OF THE INFLUENCE OF THE NEW SULFUR-CONTAINING AMINO ACID (MUELLER) ON THE ACTIVITY OF PANCREATIC AMYLASE.*

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(Received for publication, March 1, 1924.)

The work reported in this paper is a continuation of a series of studies made in this laboratory during the past few years dealing with the influence of amino acids upon the enzymic activity of various amylases (1, 2, 3). Since all the α -amino acids known to be natural products of protein hydrolysis, which have been tested here, have invariably shown, under properly controlled conditions, a favorable influence upon the saccharogenic activity of the various amylases, it was thought that it might be of interest to test in the same way the new sulfur-containing amino acid recently prepared by Mueller (4).

The work was undertaken at the suggestion of Dr. Mueller and the sample of the new amino acid used was kindly furnished by him.

The experiments were carried out in the same manner as those previously reported (1, 2), great care being taken to observe all the precautions necessary for accurate work and to have the conditions as favorable and as uniform as possible. The amino acid was tested for its influence upon both the amylolytic or starch-splitting and upon the saccharogenic or sugar-forming activity of pancreatic amylase. This was used in the form of a highly purified, very active product, No. 24 B (5).

EXPERIMENTAL.

Glycine, a typical amino acid which had previously been found to influence favorably both the amylolytic and saccharogenic

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activity of this amylase (1, 2), was used for comparison in parallel tests. In each series of experiments, determinations of the enzymic activity were made side by side upon starch dispersions containing: (a) glycine; (b) the new amino acid; and (c) no added amino acid; the latter being the "control" or standard. In order to insure uniformity of substrate 1 per cent starch dispersions were prepared by diluting a freshly prepared 2 per cent dispersion which had been properly activated and neutralized (6).

The amino acids were weighed, dissolved in a little cold water, neutralized, and added to the appropriate amounts of 2 per cent starch dispersions before making the latter up to volume. The hydrogen ion concentration of each starch dispersion was tested in an aliquot portion and adjusted to $\text{pH} = 6.9$ at room temperature. This has been found to favor the optimum activity of this amylase in digestions conducted at 40°C . for 30 minutes (6). These determinations of hydrogen ion concentration were made by the colorimetric method with Sørensen's phosphate buffer mixtures (7), which had been standardized electrometrically. The hydrogen ion concentration of the starch dispersions containing no added amino acids and of those containing glycine were also checked by the electrometric method.

Blank tests with portions of each starch dispersion but with no added enzyme were carried out under the same conditions and corresponding adjustments were made in the final weights of cuprous oxide reported.

Some typical results of a comparison of the influence of the new amino acid and of glycine on the saccharogenic activity of the amylase when tested side by side are given in Table I.

A study of the figures given in Table I shows that more cuprous oxide is obtained in the presence of the new sulfur-containing amino acid than in its absence (control tests). This indicates that the new amino acid has a favorable influence upon the sugar-forming or saccharogenic activity of the amylase. This favorable influence is not as great as that of equimolar weights of glycine and is no larger for 0.01 molar than for 0.005 molar concentrations of the amino acid. Although the differences in the weights of cuprous oxide are relatively small they are significant since they are well beyond the limits of error which have been established by many experiments for this work under the prescribed conditions.

Attention may be called to the fact that direct comparisons of weights of cuprous oxide are justified only in a given experiment where parallel tests are made side by side under the same conditions.

The results of studies of the amyloclastic activity of the amylase in which starch dispersions containing the new sulfur-containing amino acid were tested side by side with "controls" and with starch dispersions containing equimolar weights of glycine showed that the new amino acid exerts no apparent influence upon the amyloclastic or starch-splitting activity of this amylase under the conditions of our experiments. The parallel tests with glycine gave results which were typical of those previously obtained showing again that glycine influences this activity favorably.

TABLE I.

Results of a Comparison of the Influence of Glycine and of the new S-containing Amino Acid on the Saccharogenic Activity of Pancreatic Amylase No. 24 B.

	Experiment 1.	Experiment 2.	Experiment 3.
	Cu ₂ O*	Cu ₂ O*	Cu ₂ O*
	mg.	mg.	mg.
Control.....	206	216	205
New amino acid, 0.005 M.....	241	-	-
New amino acid, 0.01 M.....	-	245	238
Glycine, 0.01 M.....	278	277	268
Increase due to new acid, 0.005 M.....	+35	-	-
Increase due to new acid, 0.01 M.....	-	+29	+33
Increase due to glycine, 0.01 M.....	+72	+61	+63

*All blanks have been subtracted.

DISCUSSION AND CONCLUSIONS.

As stated above, all the α -amino acids, known to be products of protein cleavage, which have been tested in this manner, have invariably shown a favorable influence upon the saccharogenic or sugar-forming activity of pancreatic amylase. With the exception of lysine, histidine, and tryptophane the same statement may be made of their influence upon the amyloclastic activity of this amylase even when tested for only 30 minutes at 40°C. The difference in behaviour thus shown by certain amino acids is discussed elsewhere (2, 3).

Although in common with tryptophane and lysine the new sulfur-containing amino acid exerts no apparent influence upon the amylolytic or starch-splitting activity of the enzyme under the conditions of these experiments, it is found to resemble all the "natural" α -amino acids which have been tested here in favoring the saccharogenic activity of the amylase.

These results are of especial interest when compared with those previously obtained with cystine under similar conditions (2). Cystine in parallel tests with glycine was found to influence the amylolytic activity of this amylase favorably and apparently to the same extent as glycine, while this new sulfur-containing amino acid has shown no such favorable influence when tested in the same way.

It was not found possible to measure the influence of cystine upon the saccharogenic activity of the amylase by our usual method because of an interreaction of the cystine even in a concentration of 0.002 molar with the Fehling solution. On the other hand the new amino acid has shown a small but definitely favorable influence on this activity of the amylase.

As may be seen from previous reports (1, 2, 3, 8), the work in this laboratory has pointed in several different ways to the theory that this amylase either is protein in nature or has protein as an essential constituent. If this theory is correct the favorable influence of the amino acids upon the enzymic activity of the amylase and their protective action which have frequently been noted can be explained in part at least by the view that they retard the hydrolytic decomposition of the enzyme in aqueous solutions by reversing the reaction of protein changing into amino acids. From this point of view the results given above, showing that the new sulfur-containing amino acid exerts a favorable influence on the saccharogenic activity of the amylase and thus resembles the other "natural" amino acids studied, may be taken as further evidence that this amino acid may be a naturally occurring substance—a primary product of protein hydrolysis.

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ON THE ESTIMATION OF ORGANIC PHOSPHORUS.*

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In work involving the estimation of lipoid phosphorus we experienced difficulty in securing uniform results with several micro methods. Attempts to recover phosphorus from pure phosphate solutions, particularly with the Bell and Doisy (1) but also with the Bloor (2) methods, very often yielded results that were low.

Several authors have indicated that there is danger of loss of phosphoric acid during the oxidation of organic matter with acids, or in fusion methods, but the matter has apparently not been thought of sufficient importance to emphasize until recently. For example, Greenwald (3) warns against heating dry parts of ignition tubes; Raper (4), Taylor and Miller (5), and Hartwell, Bosworth, and Kellog (6) state that dry ashing is always preferable to wet ashing; Bloor (2) cautions against driving off all the sulfuric acid or too prolonged heating; and Bell and Doisy (1), Richter-Quittner (7), and others have also warned of the danger of loss of phosphoric acid by volatilization. But one is led to infer that by careful heating no loss of phosphoric acid need occur.

Recently, Lébediantzev (8) has pointed out that significant and variable losses of phosphoric acid may occur when igniting with sulfuric acid. Even more to the point is the paper of Hillebrand and Lundell (9) in which the questions of conversion of ortho- to pyro- and metaphosphoric acids and the volatilization of phosphoric acid are thoroughly canvassed. Among other things these investigators have shown that while no volatilization occurs when phosphates are heated with an excess of sulfuric acid below 150°C., prolonged heating at higher temperatures causes considerable

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loss which may amount to as much as 14 per cent; 70 mg. of P_2O_5 or more were used for each estimation. When an excess of sulfuric acid is present no conversion of ortho- to pyro- and metaphosphoric acids occurs, but if the ignition be carried to dryness, in addition to loss of orthophosphoric acid by volatilization, conversion losses to pyro- and metaphosphoric acids will result.

Loss of Phosphoric Acid by Volatilization.

Serious loss by volatilization may occur when the ashing processes of Bell and Doisy and of Bloor are used. This was shown by detecting phosphoric acid in the vapors from these ignitions.

1 cc. of a phosphate solution containing 0.1 mg. of phosphorus was placed in the bottom of Pyrex test-tubes 200×25 mm. and to them were added a few washed quartz pebbles, 8 drops of sulfuric, and 1 cc. of nitric acids as suggested by Bell and Doisy, or 1.5 cc. of the mixture of equal parts of sulfuric and nitric acids which Bloor uses. The upper parts of the tubes were then drawn out and bent at right angles and the tubes heated as directed by the respective authors. The vapors were collected by submerging the end of the tubes in dishes of water. The solutions of the distillates were concentrated on the water bath, neutralized with freshly distilled ammonium hydroxide, and the phosphorus in them was estimated.

The conditions of these experiments are not as favorable for volatilization of phosphoric acid as those which obtain in the Bell and Doisy or Bloor methods; the constriction and bend of the tube would promote condensation of vaporized phosphoric acid. Yet, even here losses of from 2 to 15 per cent were encountered.

Typical examples of many experiments are given.

	Bloor.				Bell and Doisy.	
Amount of P used, mg.....	0.200	0.200	0.100	0.100	1.00	1.00
“ “ “ in distillate.....	0.004	0.010	0.005	0.014	0.02	0.09
“ “ “ “ residue.....	0.196	0.192	0.096	0.087	0.70	0.81

Loss of Phosphoric Acid by Conversion.

In the case of the Bell and Doisy ignition it will be noted that the sum of the phosphorus in the distillate and in the residue was less than the original amount. That this additional loss was

owing to conversion of ortho- to pyrophosphoric acid was repeatedly demonstrated by igniting tubes containing pure phosphates or tissue extracts with nitric and sulfuric acids according to the directions of Bell and Doisy. Aliquots were then transferred to graduates in which the phosphorus was estimated by the colorimetric Bell and Doisy method, while other equal portions were first heated in evaporating dishes on the water bath for 2 hours, thus converting any meta and pyro acids to orthophosphoric acid. A typical experiment is given in which 0.1 mg. of phosphorus (as phosphoric acid) was used.

	mg.
Amount P used.....	0.100
“ recovered when evaporated.....	0.093
	0.090
“ “ without preliminary evaporation . . .	0.085
	0.086

Oxidation with Hydrogen Peroxide and Sulfuric Acid.

To avoid these sources of error, attempts were made to oxidize the organic matter at lower temperatures. Potassium chlorate and hydrochloric or sulfuric acids, potassium permanganate, hydrogen peroxide, and other agents were tried, and of these, heating with 20 to 30 per cent hydrogen peroxide and sulfuric acid was found to be the simplest and most efficient. With it, such difficultly oxidizable matter as fats can, in a short time and at a comparatively low temperature, be oxidized sufficiently to liberate all combined phosphorus as orthophosphoric acid. When a large percentage of fat is present, free saturated acids are formed which volatilize in the vapors of sulfuric acid. On cooling they condense; they do not interfere in the estimation. In this way phosphates can be accurately recovered and higher values obtained than when the Bloor or Bell and Doisy modifications of the Neumann ashing method are used.

Lipoid phosphorus of the blood has been determined by using the Bloor alcohol-ether extraction process, oxidizing with sulfuric acid and hydrogen peroxide, and finally the phosphorus estimated by the colorimetric method of Bell and Doisy. Lipoid phosphorus of other tissues has been determined similarly using alcohol-ether extracts of dried tissues. Total phosphorus or organic phosphorus may be estimated similarly.

Reagents.

The alcohol-ether mixture of Bloor (2) and the molybdic acid, hydroquinone, carbonate-sulfite, and phosphate solutions of Bell and Doisy (1) are used.

Distilled Hydrogen Peroxide.—This reagent is prepared from a 30 per cent solution of hydrogen peroxide.¹ About 100 cc. are distilled at a time, under diminished pressure below 60°C. The entire distillate is used. There is said to be great danger of violent explosion when working with strong solutions of hydrogen dioxide and it may produce severe burns. No trouble has ever been experienced here in working with 30 per cent solutions. *The following precautions are used:* All apparatus must be especially clean and washed finally with distilled water. The dioxide should not be allowed to come in contact with rough surfaces or organic matter and its solutions should be free from heavy metals. The distillation apparatus should be set up so that the side arm of the distilling flask and the end of the condenser project sufficiently beyond the stoppers to prevent the dioxide coming in contact with them. There is no need of using anything to prevent bumping except the usual capillary tube. According to Wolfenstein (10), hydrogen peroxide distills between 65–81°C. at 64 mm., the first fractions being low in their hydrogen peroxide content.

Description of Procedure for Estimation of Lipoid Phosphorus of Blood Plasma, Serum, or Bile, Using H₂O₂ Oxidation.

1 cc. of blood is introduced into a broad necked 50 cc. volumetric flask containing about 10 cc. of alcohol-ether mixture, shaking constantly. The mixture is brought to a boil in a water bath with continued shaking, cooled under the tap, diluted to the mark with alcohol-ether, and filtered. 20 cc. or more of filtrate containing at least 0.025 mg. of phosphorus are pipetted into a Pyrex test-tube 250 × 25 mm., 2 or 3 small, washed, glass beads added, and the extract is evaporated to dryness in a boiling water bath. It should be shaken until boiling begins and finally heated

¹ The 30 per cent hydrogen peroxide solution sold by Merck and Co. under the trade name of superoxol was used for this purpose and found very satisfactory. However, both the grades obtainable contain significant amounts of phosphoric acid.

for 10 to 15 minutes in the bath after the alcohol-ether has evaporated. 7 drops of concentrated sulfuric acid and about 0.2 cc. of 30 per cent hydrogen peroxide are then added. The tubes are covered with watch-glasses and slowly heated with a small micro burner flame, barely touching the bottom of the tube. Heating is continued until dense SO_3 fumes fill the tube, but few should be permitted to escape. The tube is allowed to cool a moment and then reheated until SO_3 fumes fully fill the tubes once again. The material sometimes chars, in which event additional hydrogen peroxide must be added, 1 or 2 drops at a time, and the process repeated until charring no longer occurs and the solution is water-clear. The watch-glass is washed with a little distilled water and the contents of the tube are transferred to a small evaporating dish and heated on the water bath for about 2 hours until all the water has evaporated. In this way traces of hydrogen peroxide which might be left, and which would interfere with the color development are driven off and any meta- or pyrophosphoric acids which may have been formed are reconverted to orthophosphoric acid.

The contents of the evaporating dish are then transferred to a 25 cc. graduate with 10 to 12 cc. of water. For standards, 5 and 10 cc. of a diluted phosphate solution containing 0.05 and 0.1 mg. of phosphorus are pipetted into 50 cc. graduates and 13 drops of sulfuric acid added to each. 1 cc. of molybdic acid and 2 cc. of hydroquinone solution are next added to the unknown and at the same time 2 cc. of molybdic acid and 4 cc. of hydroquinone to the standards and the graduates shaken. After 5 minutes when the phosphate has been changed to phosphomolybdate and reduced, 10 cc. of the carbonate-sulfite solution are added to the unknown and 20 cc. to the standards, the solutions made up to the mark and, after 8 or 10 minutes, the unknown is compared with the standards. Not more than 4 or 5 determinations should be compared in one set.

As Bell and Doisy have pointed out, the blue color formed in the alkaline sulfite medium by reduced phosphomolybdic acid changes slowly, but at different rates, in the flasks and in the colorimeter cups. It is, therefore, important to fill the standard cup with fresh standard for each unknown compared against it.

Reagents must be carefully protected from contamination. All apparatus must be thoroughly washed with good, distilled water.

Instead of using the color development of Bell and Doisy, others may be used; *e.g.*, the various strychnine molybdate colorimetric procedures, etc.

Procedure for Estimation of Lipoid Phosphorus of Tissues.

The tissue is very finely ground to a thin, uniform paste in a special hashing machine or in some other adequate way. A sample of 1 to 1.5 gm. or less is weighed out, mixed with about 3 gm. of plaster of Paris, and dried in a vacuum desiccator over sulfuric acid. The brittle mass is then thoroughly pulverized in a mortar with the aid of washed powdered glass, 60 to 80 mesh, and transferred to a Gooch crucible² on an asbestos mat or a fat-free filter paper. The mortar is washed with ether and the washings are poured through the crucible. The asbestos used should be washed with acid and extracted with alcohol and ether. The substance is then extracted in a Wiley extraction apparatus with anhydrous ether during the day (6 hours), and with anhydrous alcohol overnight. This process is repeated during the next 24 hours and, finally, it is extracted a 3rd day with alcohol. 95 per cent of the alcohol-ether-soluble material that can be extracted in 10 days is obtained in this 3 day extraction and a greater percentage of the phosphatides and cholesterol. The extract is concentrated to a few cubic centimeters and dried overnight in a vacuum desiccator over sulfuric acid. The fatty material is dissolved in anhydrous chloroform and filtered several times through a 5.5 cm. fat-free filter paper to remove any plaster of Paris that may have gone through the asbestos mat. When a clear filtrate has been obtained, the filter is immediately washed with hot chloroform several times. At this point the weight of the total ether-alcohol extract may be obtained by evaporating nearly to dryness, the last traces of solvent being removed by placing in a vacuum desiccator over sulfuric acid for 15 hours.

The fatty material is dissolved in chloroform, transferred to a graduate, and made up to 25 cc. 2 to 5 cc. are pipetted into a large Pyrex test-tube and the same process is employed as de-

² Creeping was very satisfactorily prevented by using Gooch² crucibles having the shape of a truncated cone. They were made of sheet tin (old ether cans can be used) and the diameters on top and bottom were approximately 17 and 25 mm., respectively.

scribed for blood except that usually more hydrogen peroxide is needed for the oxidation.

Comparison of Methods.

A comparison of the proposed micro estimation with a macro determination of phosphorus in yeast nucleic acid (impure) follows.

Gravimetric.	Proposed colorimetric method.					
	1	2	3	4	5	6
7.22	7.39	7.42	7.15	7.28	7.30	7.31
7.37	7.31	7.24	7.23	7.28	7.44	

Recovery of phosphorus from pure phosphate solutions has repeatedly been accomplished without ever experiencing any difficulty.

A comparison of analyses of extracts by the nitric acid oxidation of Bell and Doisy and by the hydrogen peroxide oxidation is given below.

	H ₂ O ₂ oxidation.	Bell and Doisy oxidation.
	mg. P	mg. P
Tissue extracts.	0.080	0.068
	0.080	0.072
	3.10	2.45
	3.10	2.67
	1.50	1.40
	1.50	1.32
	0.110	0.105
	0.308	0.306
	0.364	0.345
	0.299	0.305
	0.283	0.238
	0.153	0.140
	0.235	0.230
	0.146	0.136
Blood extracts.	0.054	0.050
	0.021	0.020
	0.027	0.024
1.0 mg. sodium phosphate.	1.01	0.093
0.2 " " "	0.20	0.16

SUMMARY.

1. It has been shown that serious losses of phosphorus may occur in the wet ashing used in the micro methods for estimating phosphorus due to: (a) Volatilization of phosphoric acid; and (b) Conversion of orthophosphoric to meta- and pyrophosphoric acids.

2. A process has been developed to avoid these losses. It allows from 0.02 mg. to several milligrams of phosphorus to be estimated accurately and easily.

3. Applications of the method for the estimation of lipid phosphorus in animal tissues and fluids are described, in which the error is from 2 to 5 per cent. The greatest source of error lies in the extraction processes.

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SYNTHESIS OF AMINO ACIDS IN THE ANIMAL ORGANISM.

III. CONCERNING THE SYNTHESIS OF CYSTINE IN THE BODY OF THE DOG.

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We have previously shown (1) that the two amino acids, glycocoll and glutamine, can be synthesized by the human body after the ingestion of benzoic acid or phenylacetic acid, respectively, and also that the fowl is able to synthesize ornithine (2) for the detoxication of benzoic acid.

We next undertook the work on cystine to determine whether this compound might also be synthesized under the stress of bromobenzene poisoning. Jaffé (3), and simultaneously Baumann and Preusse (4), showed that dogs fed bromobenzene excreted this substance in combination with an acetylated cysteine molecule (as *p*-bromophenyl mercapturic acid). Thomas and Straczewski (5) found that the yield of this substance is proportional to the amount of meat in the diet, while Kapfhammer (6) found none of the substance in dogs maintained on a carbohydrate diet.

We planned three experiments. In the first experiment a dog maintained on a carbohydrate diet was fed bromobenzene, and along with this, various forms of organic and inorganic sulfur; namely, sodium sulfate, potassium thiocyanate, calcium sulfide, taurine, cystine, and ethyl amino mercaptan. In the second experiment we followed the same line of procedure only adding to the substances fed in the other experiment, sufficient ammonium salts of carbonic acid and acetic acid to form nitrogen in case the nitrogen might prove the limiting factor in the synthetic process. In the third experiment the ammonium salts were replaced

by gelatin, thus providing the body with amino acid nitrogen but at the same time excluding cystine from the list.

The dog chosen as the subject of the experiment, a healthy male of 15.5 kilos of body weight, was reduced to a condition of

TABLE I.

Showing the Effects of Feeding Bromobenzene together with Various Sulfur Compounds to a Dog Maintained on a Diet of Starch and Fat.

Day.	Total N.	Urea N.	Substances fed.	Total S.	Sulfate S.		Neutral S.	Bromophenyl-mercapturic acid.
					Inorganic.	Ether.		
	gm.	gm.		gm.	gm.	gm.	gm.	gm.
1	2.714	2.274		0.353	0.172	0.023	0.159	
2	2.635	2.275		0.245	0.108	0.017	0.126	
3	3.882	Lost.	.	0.304	0.145	0.026	0.132	
4	3.201	1.311		0.401	0.145	0.018	0.237	
5	3.584	2.241	2.25 gm. C_6H_5Br .	0.371	0.064	0.084	0.223	
6	5.428	3.307	2.25 " "	0.432	0.036	0.083	0.311	
7	4.467	2.840	2.25 " "	1.013	0.668	0.076	0.268	
			2.75 " Na_2SO_4 .					
8	4.540	2.651	2.25 " C_6H_5Br .	0.445	0.121	0.078	0.245	
9	3.093	2.026	2.25 " "	0.834	0.124	0.065	0.644	
			2.60 " taurine.					
10	3.423	2.114		0.249	0.053	0.054	0.144	
11	4.926	3.056	2.10 " C_6H_5Br	0.498	0.093	0.078	0.325	
			2.00 " $KSCN$.					
12	4.564	2.947		0.398	0.017	0.047	0.333	
13	4.891	3.515	2.10 " C_6H_5Br	0.611	0.242	0.071	0.298	
			1.35 " CaS .					
14	3.781	2.428		0.307	0.119	0.024	0.164	
15	4.082	2.588	2.10 " C_6H_5Br	0.563	0.113	0.083	0.366	
			1.25 " ethyl amino mercaptan.					
16	3.141	1.788		0.296	0.227	0.036	0.237	
17	3.471	2.089	2.10 gm. C_6H_5Br	0.603	0.299	0.034	0.268	1.13
			2.50 " cystine.					
18	2.765	1.128		0.388	0.172	0.041	0.174	
19	2.423	0.800		0.201	0.073	0.023	0.104	

endogenous nitrogen metabolism and then maintained on a diet of 200 gm. of starch and 25 gm. of fat, a total of 1,050 calories per day. The amount of sulfur furnished during the feeding period was each time more than twice the amount necessary for

TABLE II.

Showing the Effects of Feeding Bromobenzene, Ammonium Salts, and Various Sulfur Compounds, to a Dog Maintained on a Diet of Carbohydrate and Fat.

Day.	Total N	Urea N	Substances fed.	Total S.	Sulfate S.		Neutral S.	Bromophenyl-mercaptano acid.
					Inorganic.	Ether.		
	gm.	gm.		gm.	gm.	gm.	gm.	gm.
1	3.217	1.972		0.301	0.115	0.027	0.159	
2	2.404	1.683		0.263	0.109	0.021	0.133	
3	2.002	1.312		0.241	0.121	0.022	0.107	
4	1.898	1.023		0.263	0.130	0.020	0.113	
5	3.271	2.486	1.5 gm. C_6H_5Br 2.0 " Na_2SO_4 3.3 " ammonium acetate.	0.889	0.564	0.203	0.121	
6	2.436	1.719		0.393	0.219	0.066	0.107	
7	4.491	2.778	1.5 gm. C_6H_5Br 2.0 " taurine 2.1 " ammonium carbonate.	0.698	0.180	0.036	0.482	
8	2.544	1.801		0.402	0.167	0.199	0.039	
9	4.738	2.935	1.5 gm. C_6H_5Br 1.5 " KSCN 3.3 " ammonium acetate.	0.562	0.146	0.221	0.202	
10	4.176	2.549		0.493	0.119	0.047	0.327	
11	3.213	1.774		0.378	0.127	0.029	0.222	
12	3.664	2.017	1.5 gm. C_6H_5Br 1.0 " CaS 2.1 " ammonium carbonate.	0.746	0.428	0.041	0.276	
13	2.492	1.90		0.272	0.147	0.036	0.089	
14	3.923	2.120	1.5 gm. C_6H_5Br 0.8 " ethyl amino mercaptan 3.3 gm. ammonium acetate.	0.540	0.209	0.262	0.070	
15	3.076	1.766		0.478	0.133	0.061	0.279	
16	4.016	2.224	1.5 gm. C_6H_5Br 2.0 " cystine 2.1 " ammonium carbonate.	0.708	0.378	0.213	0.117	0.6
17	2.930	1.732		0.404	0.211	0.033	0.160	
18	2.313	1.515		0.314	0.122	0.011	0.181	

Synthesis of Cystine

TABLE III.

Showing the Effects of Feeding Bromobenzene, Gelatin, and Various Sulfur Compounds, to a Dog Kept on a Diet of Carbohydrate and Fat.

Day.	Total N.	Urea N.	Substances fed.	Total S.	Sulfate S.		Neutral S.	Bromophenyl-mercapturic acid.
					Inorganic.	Ether.		
	gm.	gm.		gm.	gm.	gm.	gm.	gm.
1	2.587	1.313		0.144	0.108	0.031	0.049	
2	4.782	2.356		0.256	0.176	0.021	0.062	
3	2.504	1.555		0.155	0.078	0.019	0.058	
4	2.826	1.643		0.139	0.029	0.021	0.090	
5	7.949	4.205	2.25 gm. C_6H_5Br 12 gm. gelatin.	0.361	0.149	0.078	0.134	
6	5.780	3.127		0.379	0.179	0.074	0.126	
7	5.563	2.652	2.25 gm. C_6H_5Br 12 gm. gelatin 2.75 gm. Na_2SO_4 .	0.807	0.583	0.090	0.124	
8	3.683	1.820		0.314	0.102	0.041	0.161	
9	4.949	2.000	2.25 gm. C_6H_5Br 12 gm. gelatin 2.0 gm. $KSCN$.	0.645	0.103	0.045	0.498	
10	3.909	2.066		0.431	0.128	0.074	0.328	
11	3.800	2.213		0.395	0.138	0.060	0.196	
12	4.086	1.688	2.1 gm. C_6H_5Br 12 gm. gelatin 1.35 gm. CaS .	0.417	0.111	0.079	0.227	
13	1.900	0.814		0.177	0.013	0.036	0.128	
14	3.084	1.273	2.1 gm. C_6H_5Br 12 gm. gelatin 2.5 gm. cystine.	0.293	0.081	0.055	0.156	0.67
15	2.627	1.241		0.357	0.169	0.047	0.141	Trace.
16	4.750	1.900	2.1 gm. C_6H_5Br 12 gm. gelatin 1.25 gm. ethyl amino mercaptan.	0.923	0.123	0.109	0.692	
17	3.000	1.163		0.325	0.024	0.051	0.250	
18	5.152	1.060	2.1 gm. C_6H_5Br 12 gm. gelatin 2.6 gm. taurine.	0.395	0.027	0.037	0.331	
19	4.752	1.080		0.257	0.033	0.032	0.191	
20	3.800	1.250		0.269	0.033	0.034	0.202	
21	2.600	0.971		0.176	0.044	0.030	0.102	

the synthesis of sufficient cysteine for the detoxication of the quantity of bromobenzene fed. Again, the amount of nitrogen furnished either as ammonium salts or as gelatin, was several times the amount required for the cysteine synthesis. Bromophenyl mercapturic acid was found in each experiment only after the feeding of cystine, proving the inability of the animal organism to synthesize this amino acid from either reduced or oxidized sulfur in the presence of sufficient nitrogen.

DISCUSSION.

This work seemed to support in every detail the conclusion of nutritional chemists, that cystine is a necessary constituent of the protein of the diet. Though their theory would seem to have been put to a severe test by the present type of experimentation, since under the strain of bromobenzene poisoning the demand for cystine or cysteine is very acute, still, even so, no evidence of the formation of cysteine was found. It is safe to say, therefore, that the animal body cannot synthesize cysteine or cystine from the waste urea nitrogen, even when all the different forms of sulfur supposed to exist in the body's tissues are furnished; in fact, not even when more than sufficient amino acid nitrogen in the form of protein material is supplied. Moreover, very significant is the fact that the body is unable to take cystine or cysteine directly from its own tissues for the purpose of detoxicating the bromobenzene, although there is always a stimulation of tissue catabolism following the ingestion of bromobenzene. This seems to indicate that the simple hydrolytic cleavage of cystine or cysteine from tissue protein is not a normal step in cellular catabolism.

The most consequential point, however, outside of the inability of the organism to synthesize cysteine from its own destroyed tissues or from the elementary substances furnished it, seems to be the fact that there is always a relatively enormous increase in the amount of ethereal sulfates after the feeding of bromobenzene. This increase ranges from 500 to 1,200 per cent. Moreover, a close scrutiny of the data seems to indicate that the increase is even greater when the animal is maintained on a non-nitrogenous diet than when cystine is fed. Text-books tell us that ethereal sulfates are formed simply at the expense of the inorganic sulfates,

or more definitely that they are formed at the cost of the exogenous sulfur. Two facts seem to contradict this view, first that cystine (exogenous sulfur) decreases rather than increases the output of ethereal sulfates, and second, that inorganic sulfates ingested (sodium sulfate) have no effect whatever on the amount of ethereal sulfates excreted, but are excreted themselves quantitatively as inorganic sulfates. Unfortunately, therefore, the problem of ethereal sulfate formation seems not to admit of so simple an explanation. It is doubtful if any or at least more than an insignificant portion of this sulfur fraction comes from exogenous sources. Indeed, it is more likely that *all* of it is derived from endogenous catabolism. It seems most probable that the foreign molecules, such as phenol, or bromobenzene, which is first oxidized in the organism to *p*-bromophenol, or any of the hundreds of other alcoholic or phenolic compounds, which are detoxicated by combination with a sulfate radical, are first joined on to a sulfur complex, and that this is then oxidized to a simple sulfate. Precisely such a *type* of intermediary catabolism is had, at least incipiently, in the formation of *p*-bromophenyl mercapturic acid. In fact, the mercapturic acid may well be an intermediary product in the formation of ethereal sulfates, first because this acid is formed *only* by the dog, and second, because it is formed in measurable quantities only after the ingestion of cystine, and is always accompanied by a corresponding decrease in ethereal sulfates. We are working on this problem and expect to report it in detail in a future paper.

SUMMARY.

We wished to determine whether the animal body is able to build cystine (or rather cysteine). Nutritional experiments had previously shown that a protein which is deficient in cystine is not a "complete protein," and hence will not support growth, indicating that the body cannot synthesize cystine. Nevertheless, under the stress of bromobenzene poisoning, when the demand for cysteine as a detoxicating agent is most urgent, it seemed just possible that the dog might be able to effect the synthesis. This was suggested by the fact that the human body is able, on a carbohydrate diet, to elaborate glycocoll and glutamine for detoxi-

cation purposes, using the waste product urea as the source of its nitrogen, and that the chicken, under the same conditions and for like purposes, can synthesize ornithine, obtaining the necessary nitrogen from the uric acid fraction. We found, however, that on a diet of carbohydrate and fat the dog could not build cysteine at the expense of his own tissues to detoxicate the bromobenzene. We then supplied different forms of sulfur, on the supposition that sulfur might be the portion which the organism could not furnish. This included sodium sulfate, potassium thiocyanate, calcium sulfide, taurine, ethyl-amino mercaptan, and cystine. Only when cystine was fed was there a synthesis of *p*-bromophenyl mercapturic acid. In a second experiment the dog received inorganic nitrogen (ammonium acetate and ammonium carbonate) in addition to the different forms of sulfur, but again no cysteine was synthesized, evidenced by the failure to excrete the mercapturic acid except when cystine itself was fed. Finally, in a third experiment, Experiment 2 was repeated, except that at each feeding of the bromobenzene the ammonium salts were replaced by 12 gm. of gelatin, to furnish the dog with amino acid nitrogen instead of the inorganic nitrogen. But results were still negative with regard to cysteine synthesis.

It was noticed that each dose of bromobenzene was followed by a five- to tenfold increase in the amount of ethereal sulfate excreted, regardless of the other substances fed; also that the feeding of cystine tended to decrease rather than increase the excretion of this type of sulfur. It is suggested that ethereal sulfates are not formed "simply at the expense of inorganic sulfates," but rather as a result of endogenous metabolism.

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THE PARTICIPATION OF INORGANIC SUBSTANCES IN CARBOHYDRATE METABOLISM.*

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The part which phosphoric acid plays in the process of breaking down the glycogen of the working muscle (1) has led us to examine the behavior of the body phosphates and of several other inorganic salts in anabolic carbohydrate processes. A favorable approach to the study of the behavior of these inorganic salts appeared to be offered by an examination of the changes produced in their concentration in the tissues as a result of the action of the recently discovered pancreatic hormone, insulin. Should the hypothesis be correct that an intermediary phosphate compound is formed during the conversion of glucose into muscle glycogen, or during the oxidation of glucose, one should find during the process a shift of the mobile inorganic phosphates from other available body stores, such as the blood serum, into the muscle tissues. A lowering of the concentration of inorganic phosphates in the blood serum should result, which in turn should produce a drop, during this period of muscle mobilization, in the urinary phosphate excretion. The chemical transformation actively occurring, with the aid of the phosphates, in the muscle tissues, would be indicated by an increase in the total muscle phosphate concentration and by an increase in the organic phosphate fraction. The latter would represent the intermediary phosphate compound. As the transformation further progresses, and the phosphate-carbohydrate compound, in its turn, is either transformed into glycogen or broken up into smaller fragments, (presumably through

* A preliminary report of this work was published in the Proceedings of the Society for Experimental Biology and Medicine (Harrop, G. A., Jr., and Benedict, E. M., *Proc. Soc. Exp. Biol. and Med.*, 1922-23, xx, 430).

the lactic acid stage), a gradual release of inorganic phosphate back into the blood stream would be expected, with a gradual restoration of the normal blood phosphate concentration. This should then result in an increased, compensatory, urinary phosphate excretion. Large phosphate shifts due to the anabolism of carbohydrate, it was anticipated, could be most strikingly demonstrated in individuals whose glycogen stores at the outset were very low, as is known to be the case in very severe human diabetes, and in fasted animals.

We will now present the data obtained from experiments planned to test the validity of the above hypothesis.

In Table I data are presented which show the change which occurs in the concentration of some of the more important inorganic constituents of the blood following the administration of insulin.¹ The data given were obtained from experiments upon several patients with severe diabetes, and upon one normal subject.² They indicate clearly the drop which occurs in the concentration of serum inorganic phosphate and serum potassium. The only present explanation which suggests itself for the lowering of the serum potassium is the possibility that a potassium salt may also be involved in the formation of the phosphate-carbohydrate compound.

¹ The insulin used in all these experiments was that known as Iletin (insulin-Lilly). The unit employed was the "clinical unit" now commonly used in this country, which is one-fourth of the unit as originally established by Banting and his coworkers.

² The following methods were used for estimating the several inorganic constituents: (a) *Sodium*: Kramer, B., and Tisdall, F. F., A simple method for the direct determination of sodium in small amounts of serum, *J. Biol. Chem.*, 1921, xlii, 467. (b) *Potassium*: Kramer, B. and Tisdall, F. F., A clinical method for the quantitative determination of potassium in small amounts of serum, *J. Biol. Chem.*, 1921, xlii, 339. (c) *Calcium*: Kramer, B., and Tisdall, F. F., A simple technique for the determination of calcium and magnesium in small amounts of serum, *J. Biol. Chem.*, 1921, xlii, 475. (d) *Phosphates*: Briggs, A. P., A modification of the Bell-Doisy phosphate method, *J. Biol. Chem.*, 1922, liii, 13.

Blood sugar was estimated by the method of Folin and Wu. In all the analyses on blood in the experiments published in this paper the material was withdrawn under oil, free from contact with air, at once chilled in ice, centrifuged, and the plasma separated under oil.

It will be noted that the concentration of sodium is normal or somewhat lower than normal, and that the administration of insulin does not affect it. There is no striking increase in the serum potassium concentration in any of the cases such as has been recorded by Blum and Aubel (2) and others. In the severe fatal case, V.P., both the initial serum potassium and serum sodium concentrations were very low. The sodium and calcium figures which we here present do not seem to require further comment.

In Table II are given the results of experiments upon rabbits, fasted for 48 hours. The protocols of the experiments upon Rabbits A and B show the fall which occurs in the concentration of serum phosphates and potassium following the injection of insulin; that upon Rabbit C gives the range of variation under like conditions of experiment in a control animal; while the protocols of the experiments upon Rabbits D and E illustrate the remarkable increase in the serum phosphate and potassium concentrations which follows the convulsions from strychnine. Such convulsions may be expected to produce just this effect, by breaking down the glycogen (and lactacidogen) of the muscle during the severe continuous muscular contractions, into lactic acid and phosphoric acid, which latter substances are then poured out into the blood stream.

Fig. 1 indicates graphically the urinary excretion of phosphates and of potassium, as influenced by the administration of insulin. The collections of urine were made every 3 hours during the daytime and the 12 hour night excretion was estimated as one specimen. The subject was a patient with moderately severe diabetes who had been free from glycosuria for several days previous to the beginning of the experiment. During this preliminary period and throughout the experimental period he was placed upon a diet which was constant not alone in total food intake, but in the composition of the individual meals as well. The time of eating each of the meals did not vary from day to day by more than 15 minutes. His bowels were normal and regular throughout the experimental period. He had not previously received insulin. The chart with its accompanying data on this experiment made under constant conditions of food and fluid intake, and of bodily activity, clearly shows the constancy of the urinary phosphate excretion for each total 24 hour period.

TABLE I.
Effects of Insulin upon Several Inorganic Constituents of the Blood Serum.

Date.	Subject.	Time.	Analyses of venous blood serum.					Remarks.
			Sugar.	Phos- phate.	Potas- sium.	Sodi- um.	Cal- cium.	
			gm. per liter	mg. P per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	
1923								
Apr. 7	B. H.	7.30 a.m. 3.00 p.m. 4.00 " 7.30 "	3.63 1.1 0.9 1.19		18.0 13.9 16.0	300 304 290	10.0 10.4 10.4	Very severe diabetic. No acidosis. Received 10 units insulin at 7.35 a.m., 25 units at 11.30 a.m., and 10 units at 7 p.m.
Apr. 9	P. B.	7.30 a.m. 11.00 " 3.00 p.m. 4.30 "	3.12 1.32 0.87 0.81		18.2 13.6	333 326	9.2 9.8	Very severe diabetic. No acidosis. Received 30 units insulin at 7.35 a.m. and 15 units at 11.30 a.m. Symptoms of hypoglycemic shock at 4 p.m.
Apr. 14	H. S.	7.30 a.m. 12.00 n. 4.00 p.m.	2.90 0.83 0.52		18.5 13.9	313 327	9.9 9.8	Very severe diabetic. No acidosis. Received 30 units insulin at 7.35 a.m. Symptoms of hypoglycemic shock at 3.30 p.m.
Apr. 21 " 22	V. P.	11.30 p.m. 12.30 a.m. 3.00 " 6.00 " 9.00 " 9.00 p.m. 3.00 a.m.	3.16 2.26 1.14 4.64 3.10 3.42	5.3 3.8 1.9 1.3	14.3 9.7 9.4 10.5	289 275 262 286		Very severe diabetic in coma. 30 units insulin at 11.45 p.m. 30 units insulin at 3.30 a.m. 15 units insulin at 9.15 a.m., 20 units at 1.20 p.m., 20 units at 4.10 p.m., and 20 units at 10 p.m. Died at 7 a.m.
Apr. 23								

Apr. 24	E. H.	7.30 p.m.	3.60	4.37	19.0	9.2	Very severe diabetic; marked acidosis; not actual coma. 30 units insulin at 11.30 p.m. and 20 units at 8 a.m.
Apr. 25		10.30 "	3.92	3.58	12.4		
		6.30 a.m.	4.82	1.5	11.1		
		11.30 "	2.68	1.2	9.6		
Apr. 26		7.30 "	2.90	2.29	8.5		Insulin, 25 units. " 25 " " 25 " " 25 " " 25 "
" 27		8.30 "	3.56	1.8	9.9		
" 28		7.30 "	2.94	1.7	9.4		
" 29		8.00 "	2.32	1.85	10.6	9.4	
" 30		10.00 "	2.78	2.2	9.4		Normal. Received 30 units insulin at 9 a.m.
June 3	G. H.	8.50 a.m.	1.08	3.81			
		11.15 "	0.80	2.92			

On the days upon which insulin was given, the excretion of urinary phosphate was reduced during the hours in which the in-

TABLE II.

Effect of insulin and of Strychnine Convulsions upon the Serum Phosphates and Potassium of Normal Fasting Rabbits.

Date.	Rabbit No.	Time.	Blood serum analyses.			Remarks.
			Sugar.	Phosphate.	Potassium.	
			gm. per liter	mg. P per 100 cc	mg. per 100 cc.	
1923						
Apr. 25	A	11.15 a.m. 12.10 p.m.	1.38 0.25	5.02 3.11	18.6 14.2	Received 10 units insulin immediately after withdrawal of blood sample. Hypoglycemic shock in 55 min.
Apr. 26	B	10.45 a.m. 12.20 p.m.	1.28 0.31	4.17 3.11	15.6 12.6	Received 10 units insulin immediately after withdrawal of blood sample. Hypoglycemic shock in 95 min.
Apr. 26	C	11.15 a.m. 12.30 p.m.	1.34 1.75	4.35 4.39	16.6 16.3	Control rabbit. No insulin. Injection of 1 cc. normal saline solution immediately after withdrawal of first blood sample.
Apr. 28	D	2.00 " 2.50 "	1.10 1.40	3.76 7.48	18.0 33.1	Received 1.5 mg. strychnine immediately after withdrawal of blood sample. Convulsion in 50 min. Second sample of blood then taken at once.
Apr. 29	E	2.10 " 3.40 "	1.25 2.44	4.17 6.73	24.1 39.0	Convulsion in 90 min.

All these animals were fasted for 48 hours before the experiments in which they were used.

sulin was actively influencing metabolism. The release of this retained phosphate and a great increase in its urinary excretion oc-

curred during the following night, when the effect of the insulin was ended. This greatly increased night excretion following each of the insulin days is in sharp contrast to the much smaller night excretion of the control day. The excretion of potassium in the

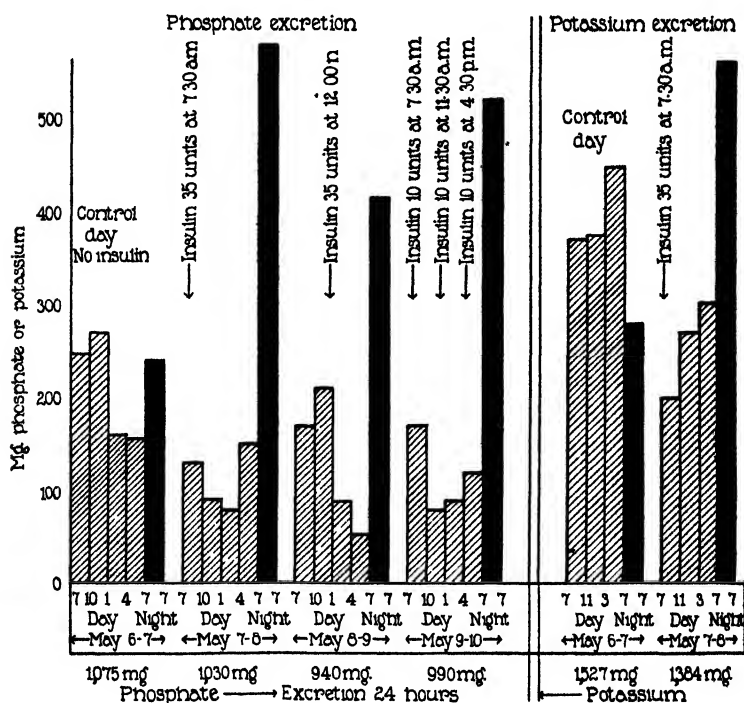


FIG. 1. The urinary excretion of phosphate and potassium following insulin administration in a diabetic.

same patient, as is indicated, showed the same variations. These variations in urinary excretion corresponded in general with like variations in the serum concentrations of phosphate and of potassium, although these changes were not striking. When the serum concentration was low, the urinary excretion was also low.

Concentration of Phosphates in the Muscle Tissues Following the Injection of Insulin.

The effect of the injection of insulin upon the concentration of the muscle phosphates has been studied in a series of experiments upon rabbits, the results of which are shown in Table III.

The method employed was similar to that used by Embden and his coworkers in their studies upon lactacidogen (3). A large number of preliminary experiments were made with chloretone, morphine, morphine and chloretone, and curare. They served to demonstrate the extreme difficulty of obtaining complete anesthesia or immobilization with any of these drugs without producing at the same time variations in the sugar and inorganic salt concentrations of the blood and muscle in control animals. Narcotics were therefore not employed.

The technique which we finally used in obtaining material for analysis in the experiments recorded in Table III was the following. The quieted animal was tied down as gently as possible, using great care to avoid pain or struggle. A small sample of blood was withdrawn from the ventricle under oil, and at once cooled and allowed to clot in ice. As soon thereafter as possible, about a gram of muscle was removed from the anterior crural region, using the greatest care to avoid pain or struggle, and to cause a minimum of bleeding. Any considerable amount of contraction of the muscle tissue such as occurs when the animal struggles will open up capillaries, and efforts were made to obtain pale material from the quiet animal. Even more important to avoid than the contamination with blood is the breaking down of the organic muscle phosphate fraction which may result from muscular contractions.

The muscle sample thus obtained was rapidly weighed in a chilled weighing bottle, and one portion ("A" sample) was placed at once in a measured quantity of ice water and well ground up with sand in a mortar immersed in a freezing mixture. The material was kept in the mixture, usually frozen solid, if it could not be analyzed at once.³ Another weighed portion ("B" sample)

³ Preservation even in a freezing mixture over a very long period causes an increase in the inorganic phosphates, doubtless due to decomposition of the organic fraction. A sample of muscle which when analyzed immediately yielded 7.3 mg. per 100 gm., yielded 8.42 mg. per 100 gm. after standing partially frozen for 3 hours and 45 minutes.

was then ground up in 1 to 2 per cent sodium bicarbonate solution, and allowed to stand at room temperature overnight before proceeding further. The maximum inorganic phosphate concentration was thus obtained and further standing did not increase it.

After being thus prepared for analysis the material was centrifuged and aliquot portions were taken for phosphate estimations by the procedure of Bell and Doisy as modified by Briggs. From 10 to 30 units of insulin were then injected into the animal and after a varying period of time, but before the onset of hypoglycemic convulsions, fresh samples of blood from the heart and of muscle from the corresponding location in the opposite intact leg were obtained, and analyzed in the same manner as above described for inorganic and for total soluble phosphates.

The glycogen of muscle juice rapidly disappears on standing, and there is a formation of lactic acid and phosphoric acid, approximately in equimolar amounts (4). This phosphoric acid is derived from the organic phosphates and reaches a final maximum in the ground muscle mixture when the latter is allowed to stand in a mildly alkaline solution. The concentration of phosphoric acid in this latter sample, accordingly, should be the total amount of organic plus inorganic phosphate present. The difference between this total amount and the amount of inorganic phosphate determined immediately in the ice-cold sample should indicate the quantity of the organic form which is present.

The distribution between the organic and inorganic fractions has not constantly led to clear-cut results, and this we attribute to technical difficulties which have not yet been overcome. Important among the disturbing factors are the muscle contractions at times met with on removal of the muscle samples. We have also repeatedly observed spontaneous fibrillary twitchings in the muscles of rabbits injected with insulin and these often occur for some time prior to the onset of the actual hypoglycemic convulsions. Because of this hyperirritability of the muscle tissues after insulin administration, the results obtained on the second muscle sample (after insulin) are apt to make the inorganic fraction appear larger than it really is in the quiet, intact muscle, and at the expense of the organic fraction. The first four experiments recorded in Table III are, we believe, essentially free from these criticisms. They indicate quite clearly the increase in the total

TABLE III.
Behavior of the Muscle Phosphates Following Injections of Insulin in Fasting Rabbits.

Date.	Rabbit No.	Weight.	Time.	Blood serum.		Muscle phosphates.						Remarks.		
				Sugar.	Phosphate.	Before insulin injection.			After insulin injection.				Increase in total phosphate.	Change in organic phosphate.
						(A ₂) Total.	(A ₁) Inorganic.	(A ₁ -A ₂) Organic.	(B ₂) Total.	(B ₁) Inorganic.	(B ₁ -B ₂) Organic.			
1923		kg.		gm. per liter	mg. per 100 gm.	mg. per 100 gm.	mg. per 100 gm.	mg. per 100 gm.	mg. per 100 gm.	mg. per 100 gm.	mg. per 100 gm.			
June 17	155	2.9	2.15 p.m. 3.45 "	1.967.68 1.154.90	15.0 12.1	15.0 12.1	2.9 2.9		18.50 14.10	4.40 3.50		Insulin, 10 units. Slight convulsions on taking second samples.		
Aug. 1	165		10.55 a.m. 12.20 p.m.	1.854.52 0.602.50	14.5 10.35	14.5 10.35	4.15 15		15.60 7.92	7.68 1.10		Insulin, 10 units. No convulsions.		
Aug. 10	168	2.6	9.25 a.m. 10.45 "	1.214.0 0.702.9	16.55 12.0	16.55 12.0	9.54 3.50		17.91 16.90	8.64 10.35	9.27 6.55	Insulin, 25 units. Insulin, 25 units. Convulsions 5 min. after taking samples.		
Aug. 28	173	2.2	1.50 p.m. 2.30 "	1.507.26 1.145.16	15.5 12.0	15.5 12.0	3.50 3.50							

Aug. 15	169	2.5	2.30 3.35	" "	1.24 1.55	4.7 4.7	13.70	7.82	5.88	13.95	8.24	5.71	0.25	-0.17	Control experiment. Second muscle sample removed 65 min. after the first. No insulin given.
Aug. 26	172	2.4	2.45 4.15	" "	1.26 0.94	8.26 8.34	15.80	7.30	8.50	17.20	9.34	7.86	1.40	-0.64	Insulin, 25 units. Convulsions before second sample could be taken. Marked fibrillary twitchings of the muscle. Second blood phosphate post mortem.
Aug. 24	171	2.0	1.50 3.50	" "	1.17 0.31	7.1 4.8	13.92	7.96	5.96	15.60	12.97	2.63	1.68	-3.33	Insulin, 25 units. Convulsions before second sample could be taken. Marked fibrillary twitching of the muscle.

phosphate content of the muscle, as well as the increase in the organic phosphate fraction, at the height of the insulin effect. In one only of these experiments (that upon Rabbit 155) is there an increase as well in the inorganic phosphate, but in this animal the increase in total phosphate was very large (from 15 to 18.5 mg. or 22 per cent), and slight convulsions were just commencing as the second muscle sample was being removed.

The data obtained in a control experiment (that upon Rabbit 169) are appended to indicate the variations which were met with when no insulin was given. The differences in phosphate concentration between the first and second muscle samples are hardly more than the extreme experimental errors of the method.

Finally, the protocols of the last two experiments appended (those upon Rabbits 172 and 171) indicate the effect of convulsions and of the preconvulsive fibrillary muscle twitchings upon the muscle phosphates. The increase in total muscle phosphate is still present, but the marked destruction of organic phosphate due to the contractions is clearly shown.

Behavior of the Serum Phosphates Following Sugar Ingestion.

It must be supposed that regulation of the output of the pancreatic hormone is normally in some way governed by the amount of material awaiting metabolism. The ingestion of food high in carbohydrate content should call out amounts of insulin adequate to the metabolic requirements, and it would be expected that the effect of the carbohydrate metabolism due to the natural hormone upon the serum concentration of phosphates would be similar to that produced by injection of the product prepared artificially. In other words, during the active metabolism of the ingested carbohydrate, a drop should occur in the concentration of serum inorganic phosphates.

In order to test this assumption, an experiment was performed upon each of three normal individuals. A blood sample was withdrawn early in the morning after the individual had been without food for a number of hours, and this was analyzed for its content of blood sugar and serum inorganic phosphate. A large quantity of glucose was then given by mouth and further blood samples were withdrawn at stated intervals, and analyzed in the same manner.

From the results of these determinations the combined blood sugar and serum inorganic phosphate curves plotted in Fig. 2 were made. The anticipated drop in the concentration of the serum inorganic

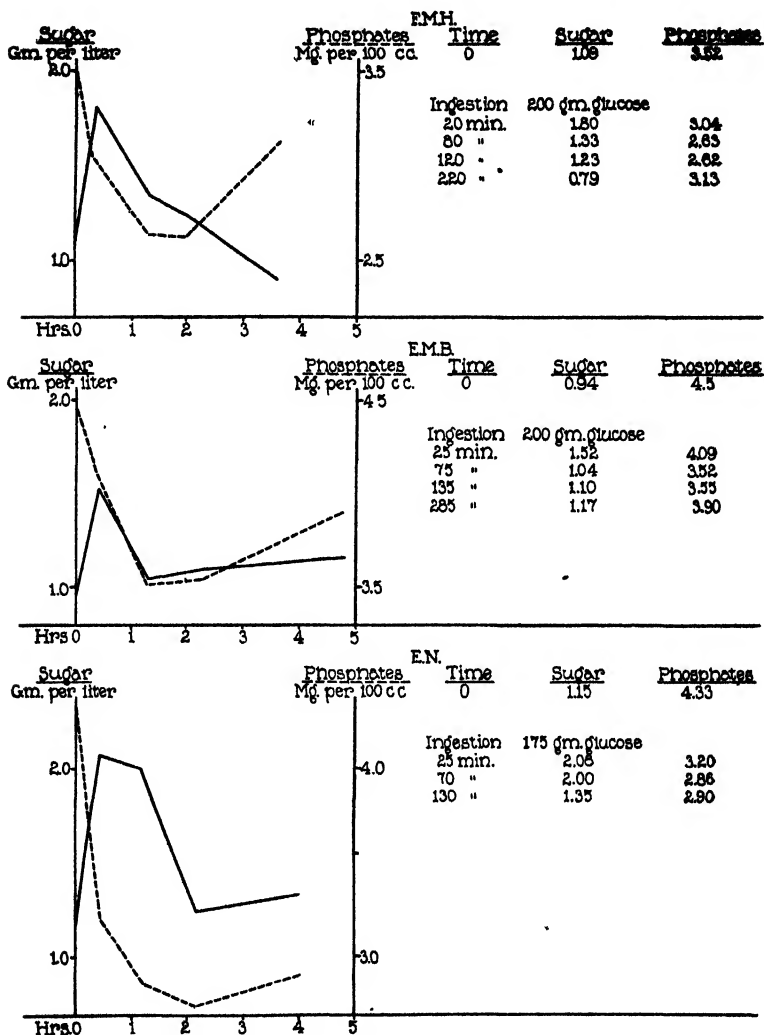


FIG. 2. The behavior of the serum phosphates following glucose ingestion in normal persons.

phosphates occurred simultaneously with the well known rise in the blood sugar concentration. Of particular interest is the fact here clearly indicated that the time of the lowest depression of the phosphates does not coincide with that of the highest level of the blood sugar concentration, but follows it. This accords in general with the interpretation of Foster (5), and of MacLean and de Wesselow (6), who consider that the rapid return of the blood sugar to its normal level, or even below after 30 to 60 minutes, is due to stimulation of the mechanism which deals with sugar (as they believe, chiefly glycogen synthesis in the liver. Absorption of glucose still continues from the intestine unchecked, but a balance is struck, as indicated by the reestablishment of the blood sugar level, while the mechanism for metabolizing the sugar continues actively at work, as indicated by the continued depression of the inorganic serum phosphate concentration. On the basis of our present view this is because the phosphates are actively employed in the conversion of carbohydrates in the tissues.

DISCUSSION AND SUMMARY.⁴

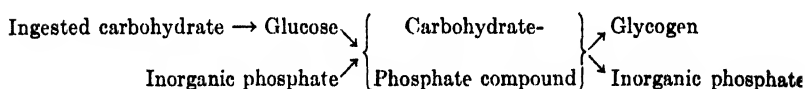
The results of these experiments indicate a shift in the mobile phosphate stores of the body during the process of active carbohydrate assimilation. There is a lessened phosphate excretion in the urine during this period, with tissue phosphate retention, which latter phosphate is later again released. One site of phosphate retention is shown to be in the muscle tissues, which are

⁴ Since the preparation of this article for the press a paper, entitled "On the effect of insulin on blood phosphate," has appeared (Wigglesworth, V. B., Woodrow, C. E., Smith W., and Winter L. B., *J. Physiol.*, 1922-23, lvii, 447). A fall in the inorganic phosphate of the blood is reported following injection of insulin, in agreement with our findings.

Date	Phosphates estimated as P.			Potassium estimated as K.			Units of insulin.
	Day.	Night.	Total.	Day.	Night.	Total.	
	mg.	mg.	mg.	mg.	mg.	mg.	
May 6-7	835	240	1,075	1,195	280	1,495	0
" 7-8	460	575	1,030	770	560	1,330	35
" 8-9	525	415	940				35
" 9-10	470	520	990				30

also one of the chief sites of carbohydrate storage. Whether liver phosphate retention also occurs, must be left to later investigation.

All the data presented are consistent with the assumption that in the storage of carbohydrate as glycogen, the intermediary aid of inorganic phosphates is required. This aid would appear to be, at least to a considerable degree, temporary in nature, because at the end of the active stage of carbohydrate anabolism, the retained phosphate is again released. If one considers the path of conversion of ingested carbohydrate into glycogen to be represented in the following scheme:



at the end of this train of events the shift must be well to the right and the store of carbohydrate-phosphate compounds probably less in amount than during the active process. The site of action of the pancreatic hormone is by no means indicated from these experiments, but the shift of phosphates following insulin injection is shown to be a constant phenomenon.

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SOME VARIATIONS IN THE ACID-BASE BALANCE OF THE BLOOD IN DISEASE.*

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In 1917 Van Slyke and Cullen¹ described a relatively simple method of measuring the so-called "alkaline reserve" of the body by estimating the plasma bicarbonate. This method quickly found extensive application in the study of pathological conditions. Van Slyke and Cullen recognized at the time that the plasma bicarbonate would serve as an adequate index of acidosis or alkalosis only as long as these conditions remained compensated, *i.e.* the C_H remained normal, but it was believed then that the C_H was a physiological constant and did not change appreciably until shortly before death. Subsequent studies by a number of different investigators showed, however, that uncompensated conditions do occur, not only in disease but also in normal individuals as a result of altered pulmonary ventilation or exercise. Van Slyke² was the first to correlate the factors involved in the normal and abnormal variations in the acid-base balance of the blood. He pointed out that there were nine theoretically possible variations, depending on the fact that the blood bicarbonate might be high, low, or normal, and in each of these conditions the pH might be high, low, or normal. As thus classified, only one condition can be considered as normal, that in which both the blood bicarbonate and pH are within normal limits.

* A report of these observations was presented at the St. Louis meeting of the American Society of Biological Chemists, December, 1923, see *J. Biol. Chem.*, 1924, lix, p. xxiii.

¹ Van Slyke, D. D., and Cullen, G. E., *J. Biol. Chem.*, 1917, xxx, 317.

² Van Slyke, D. D., *J. Biol. Chem.*, 1921, xlvi, 153.

Since the publication of this summary of the acid-base variations of the blood by Van Slyke in 1921, a number of investigations have been reported which have extended our knowledge of these variations. Increasing attention has been given to the study of the acid-base equilibrium of the blood and the literature on this subject has now become quite extensive. Two very excellent reviews of our present information of the acid-base equilibrium of the body have recently been published by Wilson³ and by The Haemoglobin Committee.⁴ We shall, therefore, refer only to the literature which is specifically connected with our present problem and make such references as may be indicated by the particular subject under discussion.

Although many conditions of abnormal acid-base balance have been reported, there has been little effort directed toward a correlation of these varying conditions into a comprehensive survey, with a uniform method of analysis. It was hoped that by such a study an abnormal acid-base balance would be disclosed in conditions where it had hitherto been unrecognized and further that the limitations of the adequacy of the bicarbonate determination might be more accurately defined.

As a measure of the acid-base balance of the blood we have estimated the pH and CO₂ content of the blood plasma. In a recent paper we have described⁵ a simple microcolorimetric technique of estimating the hydrogen ion concentration of blood plasma, based upon an adaptation of the colorimetric method of Cullen⁶ to the Myers' bicolorimeter. This method has been employed for the estimation of the pH, while the method of Van Slyke (Van Slyke and Cullen,¹ Van Slyke,⁷ and Van Slyke and Stadie⁸) has been used for the bicarbonate estimation.

The blood for each analysis was collected under oil and transferred to a specially constructed 5 cc. bulb tube and kept entirely out of contact with air throughout the manipulation. The plasma

³ Wilson, D. W., *Physiol. Rev.*, 1923, iii, 295.

⁴ The Haemoglobin Committee of the Medical Research Council, *Special Rep. Series 72*, The acid-base equilibrium of the blood, London, 1923.

⁵ Myers, V. C., Schmitz, H. W., and Booher, L. E., *J. Biol. Chem.*, 1923, lvii, 209.

⁶ Cullen, G. E., *J. Biol. Chem.*, 1922, lii, 501.

⁷ Van Slyke, D. D., *J. Biol. Chem.*, 1917, xxx, 347.

⁸ Van Slyke, D. D., and Stadie, W. C., *J. Biol. Chem.*, 1921, xlix, 1.

separated from this amount of blood was sufficient for the estimation of pH, CO_2 content, and in many instances, the CO_2 capacity. The pH was estimated within 15 minutes after the blood specimen had been collected. Inasmuch as the difference between CO_2 content and CO_2 capacity depends on the CO_2 tension of the blood at the time of its separation from the cells, the CO_2 capacity has seemed to us to add little information that could not be found from the estimation of the CO_2 content alone. While there may be some criticism of our method from the standpoint of accurate constants for the calculation of the pH and further refinement of this mathematical consideration may vary the absolute figures slightly, this will not invalidate our findings.

In our experience the pH of venous blood plasma of normal individuals at rest and at sea level falls between pH 7.35 and 7.43 and we are inclined to regard figures below 7.32 and above 7.47 as definitely abnormal. Our data, collected from a study of over 200 cases at the Post-Graduate Hospital, support the conception advanced by Van Slyke, and we have encountered conditions of acid-base variation which fall into each of the nine areas described by him. Our data also indicate that an abnormal acid-base balance is more frequently encountered than has heretofore been supposed and that there is a very practical importance in recognizing these conditions.

DISCUSSION.

In order to simplify the report of our investigation and to present the data systematically, we have classified our findings according to the nine areas outlined by Van Slyke. The data are given in Table I, the cases being arranged according to areas, from Area 1 to 9. Where the data on a given case shift from one area to another, they have all been given together in the area under which the case is discussed.

Area 1, characterized by a high pH and a high plasma bicarbonate, has been encountered after the administration of NaHCO_3 in large quantities, following x-ray treatment, as a result of loss of HCl through persistent vomiting, and from our limited experience it would seem that this condition might be associated with certain gall bladder conditions. The ratio of $\text{BHCO}_3:\text{H}_2\text{CO}_3$

TABLE I.
Data Showing Abnormal Variations in the Acid-Base Balance of the Blood.

Case No.	Name.	Age.	Sex.	Date.	Plasma pH at 38°.	Plasma CO ₂ .		Diagnosis, remarks.
						Content.	Combining power.	
Area 1.								
1	N. M.	52	F.	1923-24 Apr. 9 " 9	7.52	sol. per cent	98	Cholecystectomy, NaHCO ₃ 40 gm.; frequent vomiting. Urea N 22 mg.
						83	87	
2	F. H.	41	F.	" 10 May 3	7.45 7.49	79	79	Second analysis on Apr. 9, 6 hrs. after first. Cholecystectomy, NaHCO ₃ , frequent vomiting, temperature 102.4°F.
						76		
3	G. B.	45	M.	July 3 " 26 " 29	7.40 7.49 7.50	75	66	Gastric ulcer; Sippy treatment.
						70	68	
4	M. S.	61	M.	Oct. 29 " 31	7.53 7.53	70	70	Duodenal ulcer; Sippy treatment, discontinued Oct. 31.
						56		
5	B. W.	52	F.	Nov. 2 " 21 " 22	7.52 7.56	59	68	Urea N 10 mg. Ca of stomach, x-ray therapy, NaHCO ₃ , vomiting. Urea N 14 mg.
						62	76	
6	L. C.	62	F.	Dec. 5 " 6	7.60 7.52	75	67	Diabetes, small amount of alkali given. Acidosis diagnosed on admission, persistent vomiting, one dose NaHCO ₃ . Blood chlorides 0.438 per cent.
						70		
7	M. L.	12	"	" 17 " 17	7.52 7.53	63	63	Chronic nephritis, frequent vomiting, NaHCO ₃ , urea N 73 mg., alkali discontinued Dec. 18.
						58		
8	M. L.	52	M.	" 21	7.40			

9	D. M.	37	M.	Jan. 12	7.56	67	Hepatic abscess, postoperative, temperature 102.2°F.
10	A. C.	56	F.	" 17	7.58	84	Gastroenterostomy, temperature 101.8°F.
11	F. G.	52	M.	" 22	7.54	83	Ca of stomach, persistent vomiting. Operated Jan. 25.
				" 28	7.47	58	Blood chlorides 0.413 per cent.
12	M. K.	35	M.	" 24	7.54	88	Intestinal obstruction, persistent vomiting, blood chlorides
				" 26		104	0.320 per cent, tetany.
Areas 2 and 3.							
13	S. B.	65	F.	May 10	7.47	50	Fracture, fever 101°F.
14	I. H.	43	M.	" 10	7.47	52	Syphilis, reaction to arsphenamine, fever 102°F.
15	J. G.	57	"	" 7	7.49	61	Diabetes, fever.
16	S. L.	57	F.	Nov. 26	7.51	61	" with gangrene, temperature 102°F.
				" 26	7.51	61	Fever 102°F.
				Dec. 6	7.47	57	" 100.8°F.
Area							
17	G. B.	45	M.	July 3	7.40	75	Gastric ulcer; Sippy treatment.
18	J. S.	9	"	Apr. 13	7.41	67	Chorea.
19	D. M.	19	F.	July 8	7.43	66	Acute rheumatic fever, temperature 101°F.
20	L. C.	26	M.	" 4	7.34	69*	Epilepsy.
21	L. I.	30	"	Nov. 22	7.38	72	Duodenal ulcer; Sippy treatment.
22	B. A.	30	"	" 19	7.43	66	" " "
23	J. D.	34	"	" 19	7.41	66	" " "
24	B. A.	30	"	" 15	7.41	66	" " 2nd day tertiary phosphates.
25	J. L.	28	"	Oct. 19	7.39	71	Gastric ulcer, alkali before admission.
26	H. S.	29	"	Dec. 20	7.45	64	Hodgkin's disease, after radium treatment.

Case No.	Name.	Age.	Sex.	Date.	Plasma pH at 38°	Plasma CO ₂ .		Diagnosis, remarks.
						Content.	Combining power.	
				1923-24		vol. per cent	vol. per cent	
27	S. G.	22	M.	Mar. 28	7.34	47	28	Diabetes, 4 hrs. after insulin.
28	R. S.	49	F.	Apr. 18	7.36	47		" insulin treatment.
29	W. T.	35	M.	" 25	7.41	46		
30	E. S.	73	"	May 15	7.34	41		Chronic nephritis, urea N 46 mg.
31	G. P.	25	"	" 22	7.40	37		Ca of prostate.
				" 29	7.37	49		Diabetes.
32	M. B.	67	M.	June 13	7.36	42	44	" and pulmonary tuberculosis.
33	E. H.	50	F.	July 3	7.36	49	49	" blood sugar 0.139 per cent.
34	A. W.	51	M.	" 4	7.44	29	34	" insulin and NaHCO ₃ .
35	E. C.	39	"	" 4	7.36	41	43	Myocardial insufficiency.
36	C. W.		"	Sept. 8	7.40	49	50	Nephritis, urea N 60 mg.
37	F. P.	31	"	" 10	7.37	33	37	" " 105 "
38	S. K.	46	"	Oct. 22	7.39	37	42	" " 61 "
39	H. B.	65	F.	Nov. 12	7.38	37	43	Uremia " " 93 "
40	H. B.	14	M.	Jan. 14	7.42	53		Nephritis " " 28 "
41	A. M.	53	"	" 15	7.42	49		Cardiorenal disease.
42	S.		F.	" 19	7.42	49		Cardiac decompensation.

Areas 7 and 8.

43	E. L.	47	F.	Apr. 20	7.30	63	Cardiac decompensation.
				" 23	7.40	63	" " improved.
44	W. H.	50	M.	May 8	7.28	62	Prostatic hypertrophy.
				" 9	7.28	57	" " "
				" 14	7.30	56	" " "
				" 21	7.35	58	" " "
45	C. G.		M.	June 8	7.31	57	Diabetes, after alkali.
46	C. C.	23	"	" 9	7.33	63	
47	P.	48	"	" 11	7.33	58	
48	L. A.	66	"	" 13	7.33	62	Chronic tuberculosis, auricular fibrillation.

Area 9.

49	S. G.	22	M.	Mar. 28	7.24	22	Diabetes, blood sugar 0.288 per cent.
				" 28	7.34	28	" " 4 hrs. after insulin.
				" 29	7.29	30	" " after insulin injection.
50	J. B.	6	M.	Apr. 18	7.36	47	" " insulin treatment.
51	R. M.	45	"	Mar. 30	7.22	21	" " died few hrs. later,
52	W. T.	35	"	Apr. 5	7.15	20	Chronic nephritis, cardiac, hypertension.
				" 17	7.32		" " " "
				" 27	7.34	46	" " " "
53	M. R.	58	F.	" 27	7.22	47	Ca of breast, metastasis in lung.
54	D. D.	32	M.	" 19	7.27	33	Diabetes.
55	T. G.	28	"	" 24	7.16	22	Chronic nephritis, urea N 135 mg.
				" 30	7.22	25	" " "
				May 4	7.28	35	" " urea N 111 mg.
				" 11	7.27	46	" " " 98 "
				" 19	7.28	26	" " " 132 " Died May 19.

TABLE I—Continued.

Case No.	Name.	Age.	Sex.	Date.	Plasma pH at 38°.	Plasma CO ₂ .		Diagnosis, remarks.
						Content.	Combining power.	
Area 9—Continued.								
56	S. B.	7½	M.	1925-24 May 8 " 18 " 25 " 28 " 30 June 1 " 2	7.15 7.25 7.22 7.16 7.18 7.06 7.05	sol. per cent	sol. per cent	Chronic nephritis, urea N 89 mg. " " " " urea N 86 mg. " " " 109 " " " " " " " " " " " Diabetes. Ca of kidney. " " " " urea N 120 mg. " " alkali given. Died June 9. Diabetic coma. Diabetes (after insulin). " (" alkali). Nephritis, urea N 53 mg. " received alkali. " urea N 107 mg. Nephrosis. Uremia. Nephritis, urea N 83 mg.
						19	25	
						20		
						20		
						21		
						17		
57	E. F.	60	F.	May 24	7.31	13	20	
						37		
58	M. I.	37	M.	June 4 " 6 " 8 " 9 " 5 " 6 " 8 July 4 " 8 Oct. 18 " 19 " 26 Jan. 21	7.17 7.08 6.98 7.01 7.13 7.28 7.31 7.26 7.30 7.14 7.32 7.30 7.30	6 4 11 10 38 57 27 32 12 36 35 42		
59	J. C.	28	M.					
60	M. H.	50	F.					
61	T. M.	52	M.					
62	L. L.		"					
63	H. B.	65	F.					
64	J. B.	37	M.					

is altered by an increase of BHCO_3 . The highest pH we have encountered pathologically was 7.60, occurring in a case of diabetes following NaHCO_3 administration. The CO_2 content of this plasma was 75. It is of interest to note that acetone was being excreted by the kidneys at this time. It seems to us to be theoretically possible that the production of acetone may be increased as a result of increased alkalinity of the blood. It is well known that a decreased hydrogen ion concentration of the blood decreases the conversion of oxyhemoglobin to hemoglobin. If this is sufficiently marked, the result is oxygen want in the tissues and consequently greater inability of the cells to oxidize fats normally.

We are unable to explain at this time the remarkable change in blood reaction following x-ray treatment. Hussey⁹ has observed a condition of uncompensated alkali excess in rabbits following x-ray exposure, an observation which we have confirmed. It should perhaps be noted that there was some vomiting in the case in question, but this was neither severe nor persistent.

In three instances a condition of uncompensated alkalosis resulted primarily from persistent vomiting. The pH values were 7.52, 7.54, and 7.54, associated with plasma CO_2 contents of 70, 88, and 83, respectively. The whole blood chlorides found simultaneously in these cases were 0.438, 0.320, and 0.413 per cent, respectively.

With NaHCO_3 therapy, we have found that the bicarbonate value invariably rises before the pH becomes abnormal. We have further observed that after the administration of NaHCO_3 has been discontinued the pH sometimes returns to normal in advance of the CO_2 value while in other instances the reverse is true.

While persistent vomiting was the actual cause of the alkalosis in several instances, emesis frequently occurred in other cases after the alkalosis had become established. Some, but not all, cases in Area 1 showed an increase of body temperature.

Areas 2 and 3 are characterized by an abnormally high pH and a normal or low CO_2 content. Cases of overventilation as a result of fever are included in these areas, the rapid loss of H_2CO_3 being the responsible factor in changing the $\text{BHCO}_3:\text{H}_2\text{CO}_3$ ratio from

⁹ Hussey, R. G., *J. Gen. Physiol.*, 1921-22, iv, 511.

its normal balance. In fevers the increase in elimination of H_2CO_3 need be only relatively small to affect this ratio. Koehler¹⁰ reported this condition in cases of fever and pointed out its similarity to the overventilation due to the immersion of individuals in warm water. Voluntary overventilation for a short period of time is said to cause a greater degree of alkalosis than has ever been reported as a condition of pathological occurrence.

The highest CO_2 we have found associated with a high pH was 88. This figure for CO_2 capacity increased to 104 shortly before death. The lowest CO_2 found associated with a high pH was 50.

We are inclined to think that alkalosis is a condition often overlooked and sometimes confused with acidosis by the clinician. We believe that a great deal of care should be exercised in the administration of alkali. In cases with impairment of renal function the administration of alkali is a dangerous procedure, unless it is accompanied by estimations of the blood bicarbonate. In one instance, a case of nephritis, a small amount of alkali was sufficient to produce an uncompensated alkalosis, accompanied by a great deal of discomfort for the patient. 3 days after the alkali therapy had been discontinued the acid-base balance returned to normal and the patient was markedly improved clinically.

Area 4 is characterized by a normal pH and a high plasma bicarbonate. This condition has been encountered after the administration of NaHCO_3 in moderate amounts over a considerable period of time. It has also been observed in cases of gastric ulcer during Sippy treatment. We have likewise followed the acid-base balance of the blood of patients with gastric ulcer receiving tertiary phosphates for the neutralization of the free HCl of the stomach. Inasmuch as these phosphates are probably not absorbed from the alimentary tract, the administration of tertiary phosphates functions only in the neutralization of free HCl of the gastric juice and does not increase the systemic alkali. In our experience,¹¹ at least, the effect of administering tertiary phosphates in moderate amounts, over a considerable period of time to patients suffering from gastric ulcer, did not result in any definite change in the acid-base balance of the blood. In one case a bicar-

¹⁰ Koehler, A. E., *Arch. Int. Med.*, 1923, xxxi, 590.

¹¹ Shattuck, H. F., Rohdenburg, E. L., and Booher, L. E., *J. Am. Med. Assn.*, 1924, lxxxii, 200.

bonate content of 75 was found associated with a normal pH, after NaHCO_3 administration. We have observed that the change from normal acid-base balance to the condition of uncompensated alkalosis of Area 1 as the result of administration of NaHCO_3 proceeds by Area 4 and returns by the same area or by Area 2. The buffering action of the blood and the extent to which H_2CO_3 can be retained to balance the increase of BHCO_3 probably determines its capacity to remain compensated.

Area 5 is the area of normal acid-base balance and needs little discussion, except to mention that in our experience the pH of normal individuals falls between 7.35 and 7.43. We are inclined to regard figures below 7.32 and above 7.47 as definitely abnormal. Our values for the bicarbonate content of the plasma in this area range from 55 to 65. It has seemed unnecessary to include our data falling in the normal Area 5.

Area 6 is a condition of compensated alkali or CO_2 deficit, the pH being normal and the CO_2 low. This area includes those cases termed compensated acidosis. This condition has been found in cases of diabetes in which the abnormal acid production is not sufficiently rapid to overtax the compensating powers of the organism. Here there is an increased formation of ammonia by the kidney to combine with the acids excreted and the urine becomes more acid. As the fixed acids displace the HCO_3 of the blood bicarbonate, the CO_2 -carrying capacity of the blood is decreased so that there is need of more rapid ventilation to rid the body of the CO_2 formed constantly. Outside of the buffering action of the proteins and phosphates of the blood, the extent of the chloride shift and the amount of hemoglobin present would seem to determine the possible degree of compensation. The lowest CO_2 content we have observed associated with a normal pH was 33, occurring in a case of diabetes after insulin and NaHCO_3 administration. Early cases of nephritis also fall into this area but soon shift to Area 9 when the elimination of acid radicles is seriously impaired.

Areas 7 and 8 are conditions of uncompensated CO_2 excess, characterized by a low pH and a high or normal CO_2 . We have found this condition of acid-base balance in cases of cardiac decompensation, impaired ventilation in the lungs, and chronic tuberculosis complicated with auricular fibrillation. This con-

dition seems to be encountered only occasionally. The change in the $\text{BHCO}_3:\text{H}_2\text{CO}_2$ ratio is due to an accumulation of H_2CO_2 in the blood. The lowest pH we have encountered in Areas 7 and 8 was 7.28, the highest CO_2 content 63, and the lowest CO_2 content 56. In the one case of cardiac decompensation the pH returned to normal with improvement of the cardiac condition while the CO_2 content remained constant.

Area 9 is a condition of uncompensated acidosis, and is characterized by a low pH and a low plasma bicarbonate. This condition results from the formation of abnormal acids beyond the power of the organism to compensate, as a result of impaired renal function to eliminate normal acids or the impaired function of the kidney to form ammonia. The first group was encountered in cases of diabetes, where β -hydroxybutyric and acetoacetic acids are formed in excess of the ability of the body to compensate by buffer action or in excess of the ability of the kidney to eliminate them with sufficient rapidity as acids or neutralized salts. As a result the BHCO_3 is attacked by the fixed acids, which displace the HCO_3 of the BHCO_3 . The rate of breathing is increased due to the decreased efficiency of the blood as a carrier of CO_2 . Cullen and Jonas¹² have recently reported observations on the influence of insulin on the acid-base balance of diabetic acidosis. They found that the plasma pH and bicarbonate returned to normal coincidentally. Our data, although somewhat more limited, seem to support this general conclusion. It is of interest to note that in some of the same cases which we have studied, Killian¹³ has found that the rise in the plasma bicarbonate, following insulin therapy, was accompanied by an almost simultaneous drop in the acetone content of the blood and in its urinary excretion. The lowest pH encountered in cases of diabetes during this investigation was 7.13, and this was associated with a plasma bicarbonate content of 10 volumes per cent.

The other group of cases given in Area 9 (see Table I) all suffered from impaired renal function. Here there is a retention of acid radicles which are eliminated by the normal kidney. It is interesting to note that two of the nephritics lived for many days in a

¹² Cullen, G. E., and Jonas, L., *J. Biol. Chem.*, 1923, lvii, 541.

¹³ Killian, J. A., *Proc. Soc. Exp. Biol. and Med.*, 1923-24, xxi, 22.

condition of uncompensated acidosis, and in one of these the pH remained at a low level throughout.

The lowest pH we have encountered in any condition was 6.98, with a CO_2 content of 4, and a CO_2 capacity of 12. This observation was made on the blood of a patient suffering from carcinoma of the kidney. We have encountered many cases with pH values from 7.25 to 7.05

In the few cases of carcinoma which we have had the opportunity of examining (11 cases), the pH fell within normal limits, except where there were other complications. This does not support the findings of Chambers,¹⁴ who found that the dialysate of the venous blood of carcinoma cases was distinctly more alkaline than that of his normal subjects.

SUMMARY.

With the method employed the pH of the venous blood plasma of normal individuals at rest and at sea level was found to fall between 7.35 and 7.43, and figures below 7.32 and above 7.47 can probably be regarded as definitely abnormal.

Venous blood plasma may possess a normal CO_2 content, while the pH is outside the probable normal limits. Normal values for the CO_2 content have been found with pH values as high as 7.51 and as low as 7.28. Likewise the pH of the blood plasma may be normal, although its CO_2 content falls outside the probable normal limits. Values for the CO_2 as high as 75 and as low as 33 have been found in association with a normal pH. Therefore, the plasma bicarbonate alone does not constitute an adequate index of the acid-base balance. However, in those cases of abnormal balance due to alkali deficit, which after all constitute much the largest clinical group, the bicarbonate appears to be entirely adequate so long as the values do not fall below 35.

Uncompensated alkalosis does not appear to be a rare condition. It has been encountered following the administration of sodium bicarbonate, following x-ray treatment, after prolonged periods of persistent vomiting, in the course of fevers, and possibly in gall bladder disease.

¹⁴ Chambers, W. H., *J. Biol. Chem.*, 1923, 1v, 229.

The uncompensated acidosis of severe diabetes promptly responds to the administration of insulin, as indicated by the rise in plasma pH and bicarbonate.

Contrary to the opinion formerly held, patients may live for a comparatively long period with low pH values.

The data reported are in harmony with the conception of the variations in the acid-base balance of the blood advanced by Van Slyke.

STROPHANTHIN.

IV. ANHYDROSTROPHANTHIDIN AND DIANHYDRO-STROPHANTHIDIN.

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In former communications¹ it has been shown that strophanthidin possesses the formula $C_{23}H_{32}O_6$ and that at least one olefinic linking occurs in the molecule. Of the 6 oxygen atoms, 2 are present in the lactone group, 1 in the carbonyl group, and, finally, at least 1 in a hydroxyl group since a monobenzoate was obtained on acylation of strophanthidin in pyridine solution. The explanation of the function of the remaining 2 oxygen atoms was left to be found and was not only of importance in itself but also in permitting conclusions as to the nature of the fundamental hydrocarbon from which strophanthidin is derived.

In the communications of Windaus and Hermanns² on cymarigenin and strophanthidin, an "anhydrocymarigenin" (or "anhydrostrophanthidin") was described to which the formula $C_{23}H_{28}O_4$ was assigned. On the basis of these authors' formulation of strophanthidin, $C_{23}H_{30}O_5$, this was presumably formed by the elimination of 1 molecule of water which might be expected from the fact that strophanthidin yields a monobenzoate. However, as we have since seen that $C_{23}H_{32}O_6$ is the correct formula of strophanthidin, it became obvious that the formation of such an "anhydrostrophanthidin" with the formula $C_{23}H_{28}O_4$ must have involved the loss of 2 molecules of water and would indicate the presence of at least two hydroxyls of which one cannot be directly acylated. It was of importance, therefore, to repeat

¹ Jacobs, W. A., and Heidelberger, M., *J. Biol. Chem.*, 1922, liv, 253, 1923, lvii, 553.

² Windaus, A., and Hermanns, L., *Ber. Chem. Ges.*, 1915, xlviii, 979, 991.

the preparation and study of this substance as well as to go more deeply into the examination of the action of acids on strophanthidin. This study has given us considerably more insight into the structure of the latter as well as a proper understanding of the so called anhydro compound of Windaus and Hermanns concerning the nature of which these workers were in error.

Contrary to the result which has followed the use of aqueous hydrochloric acid in the formation of anhydro compounds from such substances as digitoxigenin and bufotalin and the like this reagent has proved inapplicable in the case of strophanthidin. When the latter is dissolved in the concentrated acid in the cold, the solution slowly becomes an olive-green, and a resinous deposit is formed which could not be crystallized. Absolute alcoholic hydrochloric acid, however, has proved much more serviceable, but the reaction which occurs is not alone one of dehydration.

When strophanthidin was dissolved at room temperature in absolute alcohol containing 10 per cent of dry hydrogen chloride, a beautifully crystalline compound was obtained which analysis showed to be $C_{25}H_{34}O_6$. A study of the properties of this substance finally furnished a clue to its nature. It is formed by the elimination of 1 molecule of water from strophanthidin. At the same time the carbonyl group in this compound is converted into the half acetal between which and another hydroxyl water is lost with formation of an inner oxide. In other words, an ethyl glucoside of the inner oxide of anhydrostrophanthidin is formed which for the present we shall designate as the ethylal of oxidoanhydrostrophanthidin. Unlike strophanthidin, this substance no longer forms a benzoate and in neutral solution no longer reacts with ketone reagents. On boiling with dilute alkali the glucosidic grouping is not effected, but the lactone group is saponified with the formation of the acid, $C_{25}H_{36}O_6$, which in turn could readily be converted into the ester by diazomethane.

On the other hand, the ethylal is very easily hydrolyzed by acids. A few minutes boiling in 50 per cent alcohol containing 1 per cent of hydrochloric acid is sufficient for the quantitative conversion of the ethylal into the hydroxy ketone or its oxidic form, whichever the case may be. The resulting anhydrostrophanthidin possesses the formula, $C_{23}H_{30}O_5$, and now readily yields

an oxime and phenylhydrazone. It likewise forms a monobenzoate with benzoylchloride in pyridine solution and a monoacetate with acetic anhydride. This fact will be discussed further on. Contrary to strophanthidin which is dextro-rotatory ($[\alpha]_D = +44$), anhydrostrophanthidin is strongly levo-rotatory ($[\alpha]_D = -145^\circ$). The removal of water, therefore, would seem to have involved one or more asymmetric carbon atoms. Anhydrostrophanthidin as obtained from 50 per cent alcohol showed no mutarotation in alcoholic solution. It still possesses a bitter taste although in much less marked degree than strophanthidin.

If strophanthidin is boiled in absolute alcohol containing 5 per cent of hydrogen chloride, it is converted into a sparingly soluble and beautifully crystalline substance which analysis showed to possess the formula, $C_{26}H_{32}O_4$. This substance proved to be the ethylal of the inner oxide of a dianhydrostrophanthidin and owed its origin to the elimination of 2 molecules of water from strophanthidin with the simultaneous formation of the ethyl glucoside of the resulting hydroxy ketone. The resulting ethylal of oxido-dianhydrostrophanthidin possesses properties analogous to those of the previously discussed ethylal of oxidoanhydrostrophanthidin and their relationship was shown by the fact that 5 per cent absolute alcoholic hydrogen chloride also converted the latter compound into the former by removal of an additional molecule of water. The ethylal of oxidoanhydrostrophanthidin is, therefore, an intermediate stage in the formation of the ethylal of oxidodianhydrostrophanthidin. Like the former compound, the latter does not react with ketone reagents and cannot be acylated. Boiling alkali saponifies only the lactone group with formation of the acid $C_{26}H_{34}O_5$ which was further characterized by the formation of an ester with diazomethane. This acid apparently showed little tendency to revert to the lactone.

On boiling with 2 per cent hydrochloric acid in 50 per cent alcohol, the very sparingly soluble ethylal gradually dissolved, owing to cleavage of the glucosidic linking with formation of the more soluble dianhydrostrophanthidin, $C_{22}H_{28}O_4$, or its inner oxidic forms. Contrary to our experience with the preparation of anhydrostrophanthidin, the formation of this substance was found to be greatly influenced by the procedure used. If the reaction product was allowed to crystallize directly from the reac-

tion mixture the resulting substance was contaminated by appreciable amounts of the original ethylal. If, however, the reaction mixture was immediately diluted with water, the product separated at once with but traces of the ethylal. This behavior may owe its origin to the fact that an equilibrium is formed even in the 50 per cent alcoholic solution between the glucoside and the hydroxy ketone which may shift with the lowering of the temperature to the point of crystallization in favor of the former. Or even if such a shift does not follow the change in temperature, owing to its low solubility, whatever ethylal should be present in the equilibrium mixture will crystallize on cooling with simultaneous formation of additional amounts in the solution in order to maintain the equilibrium. This condition is avoided by the addition of water to the hydrolysis mixture. If pure dianhydrostrophanthidin is dissolved in hot 50 per cent alcohol containing 2 per cent of hydrochloric acid and the solution is again allowed to cool, the resulting product contains about 20 per cent of ethylal.

Under certain conditions, dianhydrostrophanthidin was found to mutarotate. It exhibits an optical behavior which varies with the solvent used for its recrystallization as well as with that used for the determination of its rotation. This optical behavior and its bearing on the structure of dianhydrostrophanthidin will be discussed in a subsequent communication.

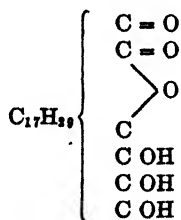
Dianhydrostrophanthidin yields both an oxime and a phenylhydrazone and what is perhaps of greater significance, as we shall discuss below, it forms an acetate and a benzoate. That these substances are not esters of the oxidic form of the hydroxy ketone was shown by their ability to form oximes. Contrary to strophanthidin and anhydrostrophanthidin the dianhydro compound no longer possesses an appreciably bitter taste, although this may be attributable in part to its lower solubility.

The previously described so called anhydrostrophanthidin of Windaus and Hermanns was obtained by the action of hydrogen chloride on strophanthidin (cymarigenin) in chloroform solution. This substance has proved to be the methylal of the inner oxide of dianhydrostrophanthidin or $C_{24}H_{30}O_4$. We have prepared the substance in accordance with the directions of these workers. After the chloroform was removed, the resulting syrup was dissolved in methyl alcohol from which the substance then

crystallized in relatively small amount. The material contained at first in the chloroform was largely a halogen compound and it is possible that a portion of it was the chloride of the inner oxide of dianhydrostrophanthidin which, when dissolved in methyl alcohol, reacted with this to form the glucoside. For comparison, the methylal was prepared directly from strophanthidin by boiling in 5 per cent absolute methyl alcoholic hydrogen chloride. The resulting methylal proved to be identical with the substance prepared by the chloroform method. The real dianhydrostrophanthidin, $C_{23}H_{28}O_4$, melts 20° lower than the methylal.

In connection with hydrogenation experiments, the dihydrostrophanthidin described in a previous communication was carried through the same series of reactions and yielded the ethylal of oxidodianhydrodihydrostrophanthidin and finally dianhydrodihydrostrophanthidin itself. Like the unhydrogenated substance the latter exhibited mutarotation under certain conditions. In the presence of palladium it slowly absorbed hydrogen. The studies on hydrogenation will be described in a later communication. In spite of the degree of unsaturation of anhydrostrophanthidin and the threefold unsaturation of dianhydrostrophanthidin, these substances react very slowly with permanganate in acetone solution. In this case, just as it has been the experience with not a few other substances, the Bayer permanganate test does not always give reliable information regarding the unsaturated character of the molecule. Hydrogenation is often a more reliable method.

From the above experiments certain conclusions may now be drawn with regard to the structure of strophanthidin. In the formula, $C_{23}H_{32}O_6$, all the oxygens have been accounted for since it is a trihydric alcohol containing in addition a carbonyl and a lactone group. This relationship may be shown by the scheme



The ring system on which strophanthidin is built consists only of carbon atoms. Since strophanthidin absorbs only 1 mol of hydrogen the basic hydrocarbon is $C_{22}H_{40}$. Since this differs from an open chain hydrocarbon by 4 mols of hydrogen it must be composed of four rings and at once justifies the suspicion of its close relationship with the hydrocarbon skeletons of cholesterol, the bile acids, and a number of other substances which are characterized by their action on the heart such as the members of the digitalis group, bufotalin, bufagin, and the like.

Certain conclusions may be provisionally made with regard to the hydroxyls in strophanthidin. Only one of its three hydroxyls has been directly acylated. Also only one of the two hydroxyls which remain in anhydrostrophanthidin yields an ester. Since dianhydrostrophanthidin still yields a benzoate and an acetate it is probable that the remaining hydroxyl which is here acylated is the same as that which is acylated in the former substances. This hydroxyl is situated in all probability in the γ position to the carbonyl group. The remaining hydroxyls are those which are readily removed as water and are apparently protected from esterification by steric hindrance. Further work is now in progress on the oxidative degradation of these substances which should throw more light on their structure and the nature of their carbon skeleton.

EXPERIMENTAL.

Ethylal of Oxidoanhydrostrophanthidin.—50 gm. of strophanthidin were dissolved in 250 gm. of absolute alcohol containing 10 per cent of dry hydrogen chloride. On standing at room temperature a gradual deposition of a hard crust of stout needles occurred. After 24 hours the separation was completed by chilling and agitating the mixture. When collected with absolute alcohol 25 gm. of crude product were obtained. This was recrystallized from absolute alcohol. On cooling, the substance separated as delicate needles which melted with decomposition at $223-230^\circ$, depending upon the rate of heating. The yield was 13 gm. The substance does not react with ketone reagents except in acetic acid solution when it gives derivatives of anhydrostrophanthidin due to hydrolysis. It is readily soluble in chloroform and acetic acid and but sparingly soluble in the cold in alcohol, methyl alcohol, benzene,

and ether. In sulfuric acid it dissolves with a bright orange-red color which deepens on standing and shows an olive fluorescence. It gives a deep olive-green with the Liebermann cholesterol reagent.

$$[\alpha]_D = -50 \text{ (c = 1.002 in CHCl}_3\text{)}.$$

Attempts to benzoylate the substance with benzoylchloride in pyridine solution resulted only in the recovery of unchanged material. Boiling alkali opens up the lactone ring without disturbing the glucosidic grouping. On boiling a suspension in 5 per cent absolute alcoholic hydrogen chloride another mol of water is removed with the formation of the ethylal of oxidodianhydrostrophanthidin to be described below.

$C_{26}H_{34}O_8$.*	Calculated.	C 72.42,	H 8.27.
	Found (a).	" 72.78,	" 8.38.
	(b).	" 72.72,	" 8.26.

Ethylal of Oxidoanhydrostrophanthidinic Acid.—4 gm. of the ethylal were saponified by boiling for 1 hour in a mixture of 400 cc. of alcohol and 300 cc. of 0.1 N NaOH. The mixture was neutralized with acetic acid and concentrated to small bulk under diminished pressure. After addition of acetic acid, a little alcohol was added, causing the separation of a gummy mass which gradually crystallized. This was collected with water and purified by suspending in water and redissolving with sufficient dilute NaOH. The filtrate was treated first with a few cubic centimeters of alcohol and then carefully with acetic acid. The initial turbidity was followed on rubbing by the deposition of characteristic rhombic plates and prisms. For analysis it was redissolved in warm alcohol and water added to crystallization. Washed with 25 per cent alcohol and air-dried it contained 1 mol of water of crystallization which could not be directly determined, since decomposition occurred when the substance was heated. At ordinary temperature in a vacuum desiccator, the substance lost no water. It begins to soften above 120° and slowly effervesces at 125°. It is

*Ethyl determinations on this substance and the dianhydro compound to be described further on were consistently low by 1 to 2 per cent even when acetic acid was added to aid solution.

readily soluble in alcohol, acetone, chloroform and ether and less readily in benzene. In sulfuric acid it forms a brown-red solution.

$C_{26}H_{36}O_6 \cdot H_2O$.	Calculated.	C 66.62, H 8.50.
	Found (a).	" 66.59, " 8.31.
	(b).	" 66.43, " 8.29.

The Methyl Ester.—The above acid was treated in acetone solution with diazomethane. After removal of the solvent and addition of alcohol, the ester separated as a compact group of highly refracting rhombs. Recrystallized again from alcohol, it sintered about 178° and slowly melted from 182 – 186° . It is soluble in the usual organic solvents except ligroin.

	$[\alpha]_D^{25} = -81.7^\circ$ ($c = 1.003$ in $CHCl_3$).
$C_{26}H_{38}O_6$.	(calculated. C 69.91, H 8.58.
	Found (a). " 70.30, " 8.59.
	(b). " 70.23, " 8.57.

Anhydrostrophanthidin.—13 gm. of the previously described ethylal were boiled in 600 cc. of 50 per cent alcohol, containing 1 per cent of hydrochloric acid, for 10 minutes. Solution occurred at once. Needles separated very slowly from the chilled solution. After standing 24 hours in the cold the crystals were collected with 50 per cent alcohol. The yield was 10 gm. Recrystallized from 50 per cent alcohol in which it easily forms supersaturated solutions it slowly separated again as needles which contained 2 mols of water of crystallization. The air-dry substance melts after slight preliminary sintering at 223 – 226° with effervescence. When recrystallized from methyl alcohol it forms slender needles which contain 1 mol of solvent which is only incompletely removed at 100° under reduced pressure. It is soluble, particularly on warming in alcohol, acetone, and acetic acid and very sparingly in chloroform, benzene, and ether. With the Liebermann test it gives a slowly developing olive-green coloration. In sulfuric acid it forms a deep red solution with a brownish reflex. Although the substance has another olefinic linking, like strophanthidin, it decolorizes permanganate in acetone solution very slowly. Likewise, in the case of strophanthidin, only one of the hydroxyl groups can be directly acylated. It still has a bitter taste which, however, is not nearly as pronounced as that of strophanthidin. The

taste develops much more gradually and is especially noticeable towards the root of the tongue. In alcoholic solution no evidence of mutarotation was observed.

$[\alpha]_D^{25} = -145^\circ$ (0.0922 gm. in 10 cc. of 95 per cent alcohol).

Air-Dry Substance from 50 Per Cent Alcohol. Dried at 100° and 15 mm. over H_2SO_4 .

$C_{23}H_{30}O_5 \cdot 2H_2O$. Calculated. H_2O 8.53.
Found. " 8.58.

Anhydrous Substance. "

$C_{23}H_{30}O_5$. Calculated. C 71.46, H 7.83.
Found. " 71.66, " 8.05.

Air-Dry Substance from Methyl Alcohol.

$C_{23}H_{30}O_5 \cdot CH_3OH$. Calculated. OCH_3 7.66.
Found. " 7.28.

Anhydrostrophanthidinoxime.—1 gm. of anhydrostrophanthidin, 0.5 gm. of hydroxylaminehydrochloride, and 2 gm. of sodium acetate were boiled for 2 hours in 20 cc. of alcohol. On dilution with water the substance slowly separated. Recrystallized from 95 per cent alcohol it formed small, highly refracting prisms which contained 1 mol of water of crystallization and melted with preliminary sintering at 260 – 265° . It is soluble in acetic acid, less readily in acetone and alcohol, and very sparingly in benzene, chloroform, and ether. The same substance was obtained directly from the ethylal and hydroxylamine in acetic acid solution, owing to cleavage of the acetal group.

Air-Dry Substance. Dried at 100° and 15 mm. over H_2SO_4 .

$C_{23}H_{31}O_5N \cdot H_2O$. Calculated. H_2O 4.30.
Found. " 4.39.

Anhydrous Substance.

$C_{23}H_{31}O_5N$. Calculated. C 68.78, H 7.79.
Found. " 68.88, " 7.84.

Phenylhydrazone.—This substance was obtained indirectly from the ethylal. 2 gm. of the latter were heated in 20 cc. of acetic acid with 2 gm. of phenylhydrazine for $\frac{1}{2}$ hour on the water bath when solution slowly occurred. After removal of the acetic acid under diminished pressure the residue was dissolved in alcohol when the phenylhydrazone quickly separated. Recrystallized from alcohol, it formed delicate needles which contained when air-dry 2 mols of water of crystallization. The substance melts slowly at 260 – 264° and is readily soluble in chloroform, alcohol, and acetone.

Air-Dry Substance. Dried at 100° and 15 mm. over H_2SO_4 .

$\text{C}_{28}\text{H}_{36}\text{O}_4\text{N}_2 \cdot 2\text{H}_2\text{O}$. Calculated. H_2O 7.03.

Found. " 6.97.

Anhydrous Substance.

$\text{C}_{28}\text{H}_{34}\text{O}_4\text{N}_2$. Calculated. C 73.06, H 7.62.

Found (a). " 73.05, " 7.43.

(b). " 73.33, " 7.45.

Anhydrostrophanthidin Acetate.—Anhydrostrophanthidin was boiled for 15 minutes in 5 parts of acetic anhydride during which the acetate crystallized. The collected product was recrystallized from alcohol. Owing to its very sparing solubility the substance required an unusual volume of hot solvent. It separated on cooling as clusters of delicate needles which sinter at 280° and melt at 292–294°.

It is readily soluble in chloroform, less easily so in acetone, and in the other usual solvents it is very sparingly soluble. Its very sparing solubility in hot alcohol is rather remarkable in view of the greater solubility of the benzoate and also of the acetate of the dianhydrostrophanthidin to be described later. In spite of the presence of two hydroxyls in the starting material only a monoacetate was found. This fact was established by the saponification figure.

0.1265 gm. of substance was boiled for 1 hour in 15 cc. of 0.1 N NaOH and 15 cc. of alcohol and the mixture titrated back against phenolphthalein.

Calculated for $\text{C}_{28}\text{H}_{32}\text{O}_6$, 2 equivalents, 5.9 cc. Found, 6.2 cc.

$\text{C}_{28}\text{H}_{32}\text{O}_6$. Calculated. C 70.05, H 7.53.

Found. " 70.22, " 7.74.

Anhydrostrophanthidin Benzoate.—1 gm. of anhydrostrophanthidin was dissolved in 15 cc. of pyridine and benzoylated with 3 cc. of benzoylchloride and the product was isolated in the usual manner. Lustrous, flat needles and platelets were formed on recrystallization from alcohol. The benzoate sinters about 270° and melts at 287–289°. It is readily soluble in chloroform and otherwise but very sparingly soluble.

0.1252 gm. of substance was boiled for 1 hour in a mixture of 15 cc. of 0.1 N NaOH and 15 cc. of alcohol which was then titrated back against phenolphthalein.

Calculated for $\text{C}_{30}\text{H}_{34}\text{O}_6$, 2 equivalents, 5.15 cc. Found, 5.60 cc.

$\text{C}_{30}\text{H}_{34}\text{O}_6$. Calculated. C 73.43, H 6.99.

Found. " 73.78, " 7.15.

Ethylal of Oxidodianhydrostrophanthidin.—100 gm. of dry strophanthidin were dissolved in 500 gm. of a 5 per cent solution of hydrochloric acid in absolute alcohol. The clear solution was refluxed for $\frac{1}{2}$ hour and towards the end the ether separated, forming a thick mass of long needles. After chilling the mixture, the substance was at once collected and washed with absolute alcohol. The crude product which weighed about 50 gm. was recrystallized from 95 per cent alcohol. About 4.5 liters were required for the operation and 40 gm. of colorless needles were obtained. The yield of substance is greatly dependent upon the care with which moisture has been excluded during the preparation of the absolute alcoholic hydrochloric acid solution. To insure sufficient drying, it has been found advisable to pass the hydrochloric acid gas through four wash bottles containing sulfuric acid. In experiments where proper precautions were not observed, the yield was greatly diminished and occasionally a second, more slowly crystallizing fraction was obtained in small amount from the crude mother liquor which consisted mainly of the previously described ethylal of oxidoanhydrostrophanthidin. If 95 per cent alcohol was used but negligible amounts of crystalline material could be recovered from the reaction mixture.

The ethylal, when rapidly heated, sinters about 238° and melts at $249\text{--}251^{\circ}$. The melting point was found to vary somewhat with different preparations, with the rate of heating, and also with the age of the preparation. The substance is easily soluble in chloroform, less readily so in benzene and acetone, and very sparingly soluble in ether. In sulfuric acid it gives a bright brown-red color and also a positive Liebermann cholesterol reaction which develops more readily than in the case of dianhydrostrophanthidin itself. As in the case of the ethylal of anhydrostrophanthidin the glucosidic linking is stable towards alkalies, but is readily hydrolyzed by dilute mineral acids.

$$[\alpha]_D^{25} = -142^{\circ} \text{ (0.5009 gm. dissolved in } \text{CHCl}_3 \text{. Volume, 10 cc.)}$$

The absence of a free hydroxyl group was indicated by the failure of the substance to yield an acetate with acetic anhydride or a benzoate with benzoylchloride. If the substance was boiled with acetic anhydride, partial decomposition occurred but unchanged material was in part recovered. If to a suspension of the substance

in acetic anhydride, sulfuric acid was added drop by drop, a reaction occurred, but from the mixture an acetate of dianhydrostrophanthidin was obtained only in small amount. The ethylal likewise failed to react in neutral solution with ketone reagents, but when the reaction occurred in acetic acid, the oxime and phenylhydrazone of dianhydrostrophanthidin were obtained due to hydrolysis of the ethylal group.

$C_{26}H_{32}O_4$.	Calculated.	C 75.71, H 8.14.
	Found (a).	" 75.94, " 8.05.
	(b).	" 75.73, " 8.15.

This substance was also obtained from the ethylal of oxidoanhydrostrophanthidin as follows: 1.7 gm. of the latter were boiled under a reflux with 17 gm. of 5 per cent absolute alcoholic hydrogen chloride without any apparent solution having occurred. The crystals slowly changed in appearance, becoming long needles. After $1\frac{1}{2}$ hours the mixture was chilled. The collected crystals were recrystallized from alcohol and agreed in all properties with the substance obtained directly from strophanthidin. It melted at $243-246^\circ$ after preliminary sintering.

$[\alpha]_D^{25} = -137.5^\circ$	(0.1025 gm. in chloroform. Volume, 10 cc.).
Found.	C 75.65, H 8.32.

Ethylal of Oxidodianhydrostrophanthidinic Acid.—8 gm. of the above lactone were refluxed for 1 hour in a mixture of 600 cc. of 0.1 N sodium hydroxide and 800 cc. of alcohol. On acidification with acetic acid, the acid separated incompletely. The remainder was obtained from the mother liquor on removal of the alcohol under diminished pressure. Recrystallization was effected by solution in warm 50 per cent alcohol with the aid of sufficient sodium hydroxide solution and reacidification. A voluminous mass of delicate needles formed. For analysis the acid was finally recrystallized by dilution of its hot alcoholic solution with water. The substance melts and decomposes at $198-200^\circ$ after preliminary sintering. It is readily soluble in chloroform, alcohol, and acetone and less readily soluble in benzene and ether. It gives a red color with sulfuric acid.

$[\alpha]_D^{26} = -85.5^\circ$	(0.1006 gm. in chloroform. Volume, 10 cc.).
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The acid shows no tendency to revert to the original lactone.

$C_{25}H_{34}O_6$.	Calculated.	C 72.42, H 8.27, OC_2H_5 10.86.
	Found (a).	" 72.50, " 8.14.
	(b).	" 72.37, " 8.32.
	(c).	OC_2H_5 10.56.

The Methyl Ester.—This ester was prepared from the acid by the action of diazomethane in acetone solution. Recrystallized from methyl alcohol, it formed colorless needles which melted at 152–156° with preliminary sintering. It is readily soluble in acetone, chloroform, and benzene and less readily in alcohol, ether, and ligroin. In sulfuric acid it forms a bichromate colored solution.

On saponification of the ester the original acid was regained.

$C_{26}H_{36}O_6$.	Calculated.	C 72.85, H 8.47.
	Found (a).	" 73.06, " 8.30.
	(b).	" 72.94, " 8.29.
	Calculated.	OC_2H_5 10.52, OCH_3 7.24.
	Found.	" 9.95, " 6.85.

Methylal of Oxidodianhydrostrophanthidin.—By the substitution of methyl alcohol in place of ethyl alcohol in the preparation of the ethylal, the methyl glucoside of dianhydrostrophanthidin was obtained. Recrystallized from methyl alcohol it formed long needles which sintered at 245° and melted at 252–254°. It is easily soluble in chloroform and less readily soluble in descending order in benzene, acetone, alcohol, ether, and finally in ligroin in which it is almost insoluble. In sulfuric acid, it forms at first a yellow solution which changes to orange.

$$[\alpha]_D^{24} = -131^\circ \text{ (0.5002 gm. in chloroform. Volume, 10 cc.)}$$

$C_{24}H_{30}O_4$.	Calculated.	C 75.35, H 7.91, OCH_3 8.12.
	Found.	" 75.30, " 8.01, " 8.56.

This substance is identical with the so called anhydrostrophanthidin incorrectly described by Windaus and Hermanns since the substance obtained by their method possessed the same properties. 10 gm. of strophanthidin were dissolved in 1 liter of chloroform and saturated with dry HCl gas according to these workers. After several hours the solution was washed with water to remove all free hydrochloric acid.

The chloroform layer after drying was concentrated to a viscous oil or resin which could not be made to crystallize. The mixture contained labile halogen since HCl gas was slowly liberated on exposure to moist air and on boiling with water the latter became strongly acid and gave a strong Cl test. The resinous mass which was at first but sparingly soluble in methyl alcohol rapidly dissolved on warming and remained soluble as if a reaction had occurred. On cooling, crystals slowly formed in small amount. After collection with methyl alcohol and recrystallization from the same solvent, it melted at 246–249° with preliminary sintering and showed no depression when mixed with the methylal described above. Windaus and Hermanns give 246°. It seems likely that the chloride of the inner oxide of dianhydrostrophanthidin is first formed which then reacts with methyl alcohol to form the ether. A small portion of the chloroform residue, when boiled with water and filtered, yielded in small amount, needles which were presumably dianhydrostrophanthidin.

$[\alpha]_D^{25} = -126^\circ$ (0.2006 gm. in chloroform. Volume, 10 cc.).

$C_{24}H_{30}O_4$. Calculated. C 75.35, H 7.91, OCH_3 8.12.

Found. " 75.06, " 7.92, " 8.07.

Dianhydrostrophanthidin. 15 gm. of the ethylal of oxidodianhydrostrophanthidin were boiled in 750 cc. of 50 per cent alcohol containing 2 per cent of hydrochloric acid. The ether rapidly dissolved and the boiling was continued for 45 minutes. The hot solution was at once diluted with an equal volume of water causing the immediate separation of needles which, after cooling, were collected with water. If this dilution was omitted the resulting product was found to contain in the neighborhood of 25 per cent of ethylal, which was obtained on recrystallization. Recrystallized from acetone, dianhydrostrophanthidin separated incompletely as needles and more was obtained from the mother liquor. The yield was 11 gm. When recrystallized from acetone, the substance melted at 233–236° with slight preliminary sintering and no longer gave a positive Zeisel. It is readily soluble in chloroform, less easily in acetone, and with difficulty in alcohol and benzene. It forms a deep red solution in sulfuric acid and gives a Liebermann cholestol reaction. As in the case of anhydrostrophanthidin, although it has an additional olefinic linking, it decolorizes perman-

ganate in acetone solution only slowly. Contrary to strophanthidin and anhydrostrophanthidin, it has no longer a perceptibly bitter taste, although the dust causes a bitter sensation in the nasopharynx.

$[\alpha]_D^{25} = -222^\circ$ (0.5015 gm. in alcohol-free chloroform. Volume, 25 cc.).
 $C_{23}H_{28}O_4$. Calculated. C 74.96, H 7.66.
 Found. " 74.77, " 7.72.

Oxime of Dianhydrostrophanthidin.—A solution in alcohol of the ketone with equivalent amounts of hydroxylamine hydrochloride and sodium acetate was refluxed for 2 hours and yielded small, stout, glistening prisms. When recrystallized from 95 per cent alcohol the oxime melted with effervescence at $279-281^\circ$ with preliminary sintering. It is sparingly soluble in the usual solvents and in sulfuric acid gives at first a yellow solution which changes to a deep red with brown fluorescence.

$[\alpha]_D^{25} = -174^\circ$ (c = 1.006 in pyridine).
 $C_{23}H_{27}O_4N$. Calculated. C 72.02, H 7.63.
 Found. " 72.03, " 7.59.

Phenylhydrazone of Dianhydrostrophanthidin.—1.5 gm. of dianhydrostrophanthidin and 1 gm. of phenylhydrazine dissolved in 1 cc. of acetic acid were refluxed for 2 hours in 20 cc. of alcohol. Lustrous crystals of the hydrazone slowly separated after cooling. Recrystallized from alcohol it slowly deposited as aggregates of lustrous platelets and needles which, when air-dried, contained 1.5 mols of water of crystallization. It melts slowly at $219-227^\circ$ with preliminary sintering. It is easily soluble in chloroform and acetic acid, less readily in alcohol, and but sparingly so in ether or benzene.

$[\alpha]_D^{25} = -245^\circ$ (0.1 gm. in chloroform. Volume, 10 cc.).

Air-Dry Substance. Dried at 100° and 15 mm. over H_2SO_4 .
 $C_{23}H_{34}O_4N_2 \cdot 1.5 H_2O$. Calculated. H_2O 5.56.
 Found. " 5.16.

Anhydrous Substance.

$C_{23}H_{32}O_4N_2$. Calculated. C 75.93, H 7.48.
 Found. " 75.72, " 7.69.

Dianhydrostrophanthidin Acetate.—1.5 gm. were boiled in 15 cc. of acetic anhydride for 30 minutes. The acetate crystallized on decomposition of the anhydride with water. Recrystallized from alcohol it formed arborescent aggregates of small, stout prisms containing 0.5 mol of water of crystallization. It softens above 180° and slowly shrinks to a resin which becomes fluid at 203–206°. It is easily soluble in acetone, chloroform, and benzene and less readily so in alcohol and ether. In sulfuric acid it gives a deep bichromate red solution and also a positive Liebermann reaction.

$$[\alpha]_D^{22} = -222^\circ \text{ (0.1 gm. in chloroform. Volume, 10 cc.)}$$

0.1125 gm. of air-dry substance was boiled 1 hour in 15 cc. of 0.1 N NaOH and 15 cc. of alcohol and then titrated back against phenolphthalein. 5.4 cc. of alkali were consumed. The theory for 2 equivalents required by the lactone and acetyl groups is 5.37 cc.

Air-Dry Substance. Dried at 100° and 15 mm. over H₂SO₄.

C₂₆H₃₀O₆ · 0.5 H₂O. Calculated. H₂O 2.15.

Found. " 2.28.

Anhydrous Substance.

C₂₆H₃₀O₆. Calculated. C 73.13, H 7.37.

Found. " 73.18, " 7.53.

Dianhydrostrophanthidin Benzoate.—1.5 gm. were benzoylated in the usual way in 15 cc. of pyridine with 1.5 cc. of benzoylchloride. The crude benzoate was recrystallized from alcohol and because of its sparing solubility, about a liter was required. It separated from alcohol as short prisms with square ends and from benzene in radiating groups of wedge-shaped needles. It is readily soluble in chloroform and very sparingly in the other usual solvents.

C₃₆H₃₄O₆. Calculated. C 76.23, H 6.83.

Found. " 76.18, " 6.94.

The fact that in the formation of the acetate and benzoate of dianhydrostrophanthidin the substance is acylated as a hydroxy ketone and not as the oxidic form was demonstrated by the preparation of oximes.

Oxime of Dianhydrostrophanthidin Benzoate.—0.5 gm. of the benzoate was heated with 0.5 gm. of hydroxylamine hydrochloride and 2 gm. of sodium acetate in 30 cc. of acetic acid. On cooling, glistening prisms formed which were increased by dilution with

water. The substance recrystallized from alcohol formed small, glistening rhombs and prisms which melted with decomposition at 283–285°, and were very sparingly soluble in all solvents.

$C_{10}H_{13}O_5N$. Calculated. C 73.88, H 6.83.
Found. " 73.57, " 6.87.

Oxime of Dianhydrostrophanthidin Acetate.—This was prepared in the usual manner in alcoholic solution. It forms four-sided platelets from alcohol which melt at 224–226° with preliminary sintering.

$C_{10}H_{11}O_4N$. Calculated. C 70.54, H 7.35.
Found. " 70.74, " 7.70.

Ethylal of Oxidodianhydrodihydrostrophanthidin.—16 gm. of anhydrous dihydrostrophanthidin were boiled in 80 gm. of absolute alcohol, containing 5 per cent of dry hydrogen chloride. The ethylal formed needles on cooling which were collected with absolute alcohol and recrystallized from alcohol. The substance sinters above 210° and melts at 216–218°. It is easily soluble in chloroform, acetone, and benzene, and somewhat less soluble in cold alcohol and ether. It gives the Liebermann cholesterol reaction. In sulfuric acid it forms at first a brown solution which rapidly changes to a red.

$[\alpha]_D^{25} = -101.3^\circ$ (0.1066 gm. in chloroform. Volume, 10 cc.).

$C_{25}H_{34}O_4$. Calculated. C 75.33, H 8.61.
Found (a). " 75.12, " 8.44.
(b). " 75.23, " 8.60.

Dianhydrodihydrostrophanthidin.—3.8 gm. of the ethylal were hydrolyzed for $\frac{1}{2}$ hour in 100 cc. of 50 per cent alcohol, containing 2 per cent of hydrochloric acid. The clear solution after dilution with water deposited rosettes of needles which were collected with water. Recrystallized from dry acetone, it forms needles which sintered at 190° and melted at 193–199°. It is readily soluble in acetone, chloroform, alcohol, and benzene, and very sparingly in ligroin. In sulfuric acid it gives at first an orange color which deepens to red on standing. In chloroform solution there was no evidence of mutarotation.

$[\alpha]_D^{25} = -184^\circ$ (0.0869 gm. in chloroform. Volume, 10 cc.).

$C_{25}H_{30}O_4$. Calculated. C 74.55, H 8.17.
Found. " 74.68, " 8.32.

Oxime of Dianhydrodihydrostrophanthidin.—1 gm. of the ketone, 0.5 gm. of hydroxylamine hydrochloride, and 2 gm. of sodium acetate when boiled in 20 cc. of alcohol readily yielded needles which when recrystallized from alcohol formed prisms which melted at 291–292°. The substance is very sparingly soluble in the usual solvents.

$C_{21}H_{31}O_4N$.	Calculated.	C 71.64,	H 8.11.
	Found.	" 71.93,	" 8.23.

THE DEPLETION OF VITAMIN C IN THE LIVER OF THE GUINEA PIG ON A SCORBUTIC RATION.*

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(Received for publication, February 4, 1924.)

The dissimilarity in the antiscorbutic requirement of the rat and the guinea pig has been frequently pointed out. This dissimilarity led to an investigation by one of us (H.T.P.) of the antiscorbutic content of the liver of the rat. The results showed a high content of antiscorbutic vitamin in the liver of the rat, which was apparently uninfluenced by the antiscorbutic content of the diet over several months' time (1). A later experiment (2) carried out with greater detail showed no perceptible decrease in the antiscorbutic content of the rat's liver, even though the rats had been restricted for two generations to a purified ration containing, at most, only traces of antiscorbutic vitamin. These early results were also confirmed by Lepkóvsky and Nelson (3) who fed a somewhat different ration over the period of two generations in the attempt to deplete the rats of vitamin C, and used smaller doses of the rat liver.

It was suggested in the first report cited (1), that:

"It is conceivable that the liver cells are so constituted that a considerable supply of the antiscorbutic factor may be contained in them while at the same time the content of other tissues is below the level at which proper functioning is possible. It is proposed to test this point by determining also the antiscorbutic content of livers of guinea pigs suffering from scurvy as contrasted with the livers of normal guinea pigs."

It was for the purpose of carrying out this phase of the problem that the present investigation was undertaken.

* Published with the permission of the Director of the Wisconsin Agricultural Experiment Station.

Based in part upon graduate work performed by the Junior author in partial fulfillment of the degree of Master of Science.

EXPERIMENTAL.

Rations.

Two scorbutic rations were used in the feeding of the guinea pigs. Ration I was the soy bean scorbutic ration used in the first investigation cited (1).

Ration I.

	<i>per cent</i>
Soy beans or soy bean flour	84
NaCl.....	3
Ca lactate.....	3
Dried yeast.....	3
Filter paper.....	2
Butter fat.....	5

For this ration, both soy bean flour (Hepco) and soy beans cooked in the pressure cooker for 1 hour at 15 pounds pressure were used. To prepare this ration, the filter paper is torn into small pieces and beaten in a quart of distilled water, until a fine pulp is obtained. This is poured on the mixed dry ingredients and rubbed until uniform. When dry it is ground or crushed and mixed thoroughly with melted butter fat. Baker's compressed yeast was used.

Ration II was that used by Steenbock and coworkers (4).

Ration II.

	<i>per cent</i>
Rolled oats.....	69
Alfalfa meal (autoclaved 30 minutes at 15 lbs. pressure).....	25
Casein.....	5
Common salt.....	1

The casein was purified following the technique outlined by Steenbock and associates (5) with the following slight modifications: Tap water was used during the whole period; and the water was changed only once each day for 7 days.

This second ration was included in the investigation for purposes of comparison, to make sure that the experimental conditions of this and other investigations were comparable. No significant differences were noted in the severity of the scurvy symptoms induced by the two diets, or the time at which these appeared. There were, however, some differences noted in the readiness with which the animals adapted themselves to the two diets, and the regularity of growth upon them.

Animals.

The determination of the concentration of the antiscorbutic vitamin in the livers of guinea pigs, fed rations varying in their content of this vitamin, was made by feeding varying doses of the livers to a group of guinea pigs (Group A) which showed the early symptoms of scurvy, including unmistakable swelling of wrists, after a preliminary period on a scorbutic diet. Guinea pigs, Nos. 102 and 106, were fed Ration I and Nos. 110, 111, 118, 121, 122, and 133 were fed Ration II. The point at which doses of liver were begun is indicated on Charts I and II.

TABLE I.

Serial No. of guinea pig.	No. of days on scorbutic diet.	Maximum weight of guinea pig.	Weight of guinea pig when killed.	Weight of liver.
		<i>gm.</i>	<i>gm.</i>	<i>gm.</i>
112	20	185	126	5
117	20	205	164	9.6
129	20	261	215	15.03
108	21	185	127	8
119	21	179	130	7.6
118	22	200	127	6.5
109	22	235	150	9.2
123	22	375	318	18.09
113	23	173	128	7.8
115	23	262	198	12.45
107	24	270	196	12
125	24	414	332	19.2
124	26	393	245	13.13
128	28	357	195	8
130	28	383	255	12

A group of guinea pigs (Group B) was fed Ration II for from 20 to 28 days. In no case was a guinea pig used until marked symptoms of scurvy had developed. Table I gives the weights of the guinea pigs and of the livers used. It was thought to be of interest to record these data since there is no close agreement in the literature as to the relative change in the weight of the liver during the period of depletion of vitamin C. The literature on the subject is reviewed, and new experimental data presented by Bessesen (6).

The other group of guinea pigs (Group C) was fed a ration high in antiscorbutic vitamin. In order to have as close a check

with Group B as possible the basal diet of Group C was identical in its percentage composition with Ration II. However, no pains were taken to autoclave the alfalfa meal or purify the casein. The presence of abundant antiscorbutic vitamin in the diet was assured by offering fresh cabbage at all times.

Preparation of Liver.

The preparation of the guinea pig livers followed closely the method previously described (2) for preparation of the rat's liver. The guinea pig, which was to furnish the liver, was chloroformed and the liver removed a short time after death ensued. One exception should be noted in the case of a pig which died with pronounced scurvy symptoms. In this case, its liver was fed within a few hours after the death of the animal. Since the guinea pig differs from the rat, in that it has a gall bladder, this was removed before the amounts to be fed were weighed out.

In feeding the livers of normal guinea pigs, the size of the livers (varying from 21 to 31.8 gm.) was such that an efficient utilization of material could best be accomplished by feeding twice the prescribed amount on alternate days. This method was utilized also in the case of Guinea Pig 133, fed a 7.5 gm. "daily" dose of scorbutic guinea pig liver. In the other instances, however, the liver of one guinea pig on the scurvy ration was sufficient to furnish the daily doses of the three guinea pigs fed.

RESULTS.

The results of the experiment are shown in Charts I and II. It will be seen that in the case of the guinea pigs given the doses of the livers of normal guinea pigs, the loss in weight was checked in from 1 to 4 days after the addition of liver was started, and the gain in weight continued for from 14 to 16 days until the autopsy was held. A corresponding increase in the activity and well-being of the pigs was also noted. The autopsy showed that scurvy symptoms had almost disappeared in every case.

The scorbutic guinea pigs, given the doses of livers of scorbutic pigs, continued to lose weight steadily until death, which ensued from 5 to 9 days after addition of liver was started. Autopsy of these pigs showed pronounced scurvy symptoms.

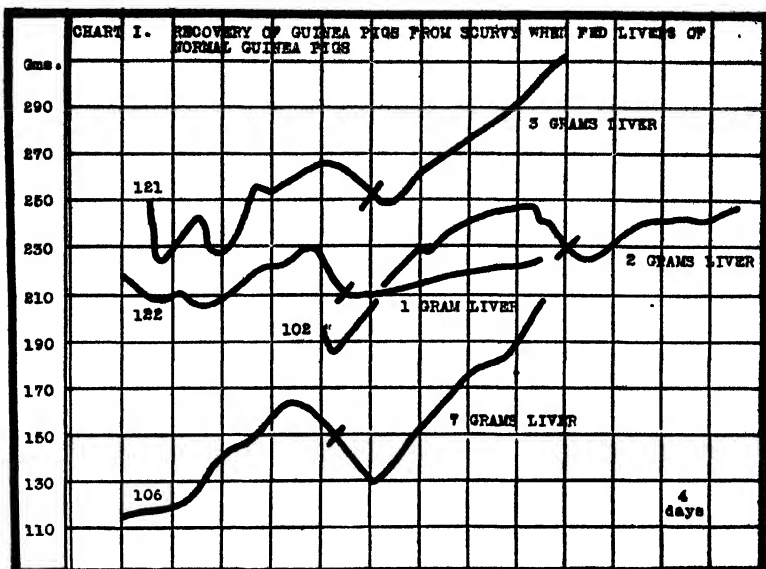


CHART I.

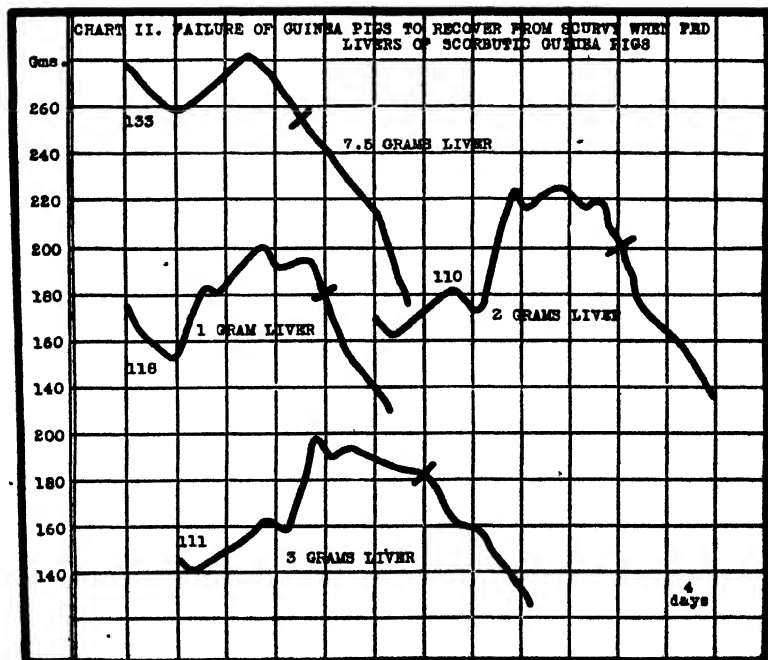


CHART I.

DISCUSSION.

From the results presented it will be seen that in the liver of the normal guinea pig, vitamin C is found in abundance; and that in the liver of the guinea pig fed a diet lacking in this factor, vitamin C becomes strikingly depleted. That the concentration of vitamin C in the normal guinea pig liver is comparable with that in the rat liver is indicated by the close similarity of the growth curves in the present study and in that of Lepkovsky and Nelson (3) wherever comparable doses of liver were used.

The depletion of vitamin C in the guinea pig's liver, when this factor is lacking in that animal's food is another example of such vitamin depletion in the liver already demonstrated in the rat in regard to vitamin A by Steenbock, Sell, and Nelson (7), and in regard to vitamin B by Osborne and Mendel (8).

The present investigation, in addition to these other two instances of depletion, renders all the more surprising the persistence of vitamin C in the liver of the rat even after long intervals on a diet containing at most only traces of this factor. It makes very improbable the hypothesis quoted at the beginning of this paper; *i.e.*, that the persistence of this factor in the rat's liver under such conditions might be accounted for by assuming that a peculiar relationship exists in the liver in regard to vitamin C whereby this factor might persist in this organ after its depletion in the rest of the body. It, therefore, again emphasizes the reasonableness of the hypothesis emphasized in the first report (1) of this series; *i.e.*, that the rat produces vitamin C in metabolism from some source not available to the guinea pig. Whether this is a true synthesis, or a conversion of some closely related compound is being investigated.

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THE ABSORPTION, DISTRIBUTION, AND ELIMINATION OF ETHYL ETHER.

I. THE AMOUNT OF ETHER ABSORBED IN RELATION TO THE CONCENTRATION INHALED AND ITS FATE IN THE BODY.

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(Received for publication, March 1, 1924.)

This series of papers deals with the quantitative aspects of the absorption, elimination, and distribution of ethyl ether in the body, and its general physiological effects. The mechanism of absorption, distribution, and elimination presented is applicable not alone to ether but to any gas or vapor which is not altered or destroyed in the body.

This investigation has been made possible through the development by the writer of a method for the analysis of ethyl ether in air and blood, by means of a train containing iodine pentoxide. The ether vapor, drawn through such a train, is oxidized and thus liberates iodine which is collected and determined by titration with thiosulfate, using starch as an indicator. In rapidity, accuracy, and ease of technique, when familiarity has been acquired, this method has great advantages over the methods which former investigators have had at their service. The method of analysis is detailed in a previous paper (1).

This paper deals with the general experimental procedures, the fundamental principles governing the physical behavior of ether vapor, and its distribution between blood and air. In addition, the total amount of ether absorbed in relation to the concentration inhaled and the fate of the ether in the body, have been determined experimentally. These data form the basis for the theoretical discussion of the mechanism of absorption and elimination presented in subsequent papers.

1. Method and Apparatus.

The experimental procedures used were sufficiently uniform throughout the investigation to allow a general description. Such special procedures as were developed are detailed with their respective experiments.

Dogs were used as the experimental animals. Under local anesthesia with cocaine, cannulas were inserted into the carotid or femoral artery from which blood was to be drawn during the experiment. A tracheotomy was performed, also under cocaine; and a cannula was inserted and attached to a pair of mica disc valves. The total dead space formed by the valves and connections closely approximated that portion of the upper respiratory tract which was short circuited by the tracheotomy.

In administering ether to the animals under study, the inspiratory valve was connected to a spirometer (it had only a narrow annular water space) of 400 liters capacity filled with the desired mixture of ether and air. This mixture was prepared by evaporating into the stream of air drawn in to fill the spirometer the approximate weight of ether necessary to make the desired concentration. After thorough diffusion had taken place a sample was drawn and analyzed and on this basis more air was drawn in and mixed until the correct concentration was shown by analysis. Throughout the period of inhalation further samples were taken at frequent intervals to confirm the maintenance of this concentration.

When once obtained the concentration in the spirometer held with little loss for several hours. Absorption of the ether in the small surface of water exposed in the spirometer was almost negligible.

The expiratory side of the respiratory valves was connected by a tube and valves to discharge into two spirometers, one of 50 liters and the other of 250 liters so that the expired air could be passed into either of the spirometers, or from the smaller into the larger. The total expired air was collected in the large spirometer over long periods of time. The small spirometer was used to trap a mixed sample of expired air at any point in the experiment. From this sample the ether content of the expired air was determined.

Both the 400 liter and the 250 liter spirometers were equipped with fans to mix the contents. The shafts driving these fans passed out of the tops of the spirometers through packing boxes and water seals. The fans were rotated by electric motors mounted on the tops and thus outside of the spirometers.

Volume readings of the contents of the spirometers were taken from a scale placed upon the side of the bells. All readings were taken with the air in the spirometers at the prevailing barometric pressure. This end was attained by means of an open end water manometer attached to the outlet tube of the spirometers. At the time the volume was read, the weight of the spirometer was manipulated until the levels of the water in the two arms of the manometer were the same. The maximum variation from atmospheric pressure at any position of the spirometers was at most only a few millimeters of water.

The spirometers used were of the "dimple" type and thus presented a minimum of water surface to the enclosed gas. Even the most carefully constructed spirometer of this type has a dead space which, although it can be flushed, requires a correction in calculating the ether concentration of the air.

This dead space was calculated by emptying the apparatus as completely as possible and then drawing in 40 liters of a known dilute mixture of CO_2 in air. After running the fan for a few minutes to mix the contents of the spirometer, a sample was drawn and analyzed for CO_2 . The ratio of the initial percentage of CO_2 to that found in the mixed air affords a basis for calculating the magnitude of the dead space.

The average of a number of similar observations gave a dead space of 107 liters for the 400 liter spirometer and 8 liters for the 250 liter spirometer. These corrections are incorporated in all calculations made for the ether content of the respired airs.

The arrangement of apparatus as described is illustrated in Fig. 1.

2. Method of Analysis.

The ether content of air and blood was determined, as already stated, by the iodine pentoxide method developed for this purpose by the author. This method as employed has a maximum error of ± 0.04 mg. of ether in the sample used for analysis. In the

present work the samples of blood used were all of 1 ml. volume; and the ether content is expressed in terms of grams per liter of blood. The figures given would thus have a maximal error of ± 0.04 gm. of ether per liter of blood. This error may be exceeded, however, by the inaccuracies involved in measuring 1 ml. of blood. Even though the pipettes used were recalibrated to deliver 1 ml. of this fluid, the variation in viscosity of blood is

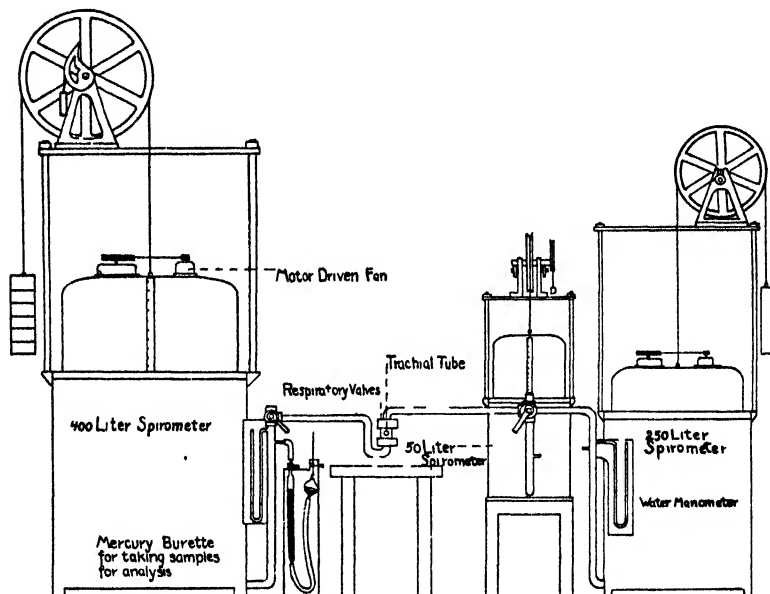


FIG. 1. Showing arrangement of apparatus employed for the quantitative study of the absorption and elimination of ether.

such that it is probable that the total accuracy of the determinations of ether in blood does not exceed ± 0.1 gm. per liter.

Great care is necessary in the manipulation of blood samples that a part of the ether is not lost, since when drawn the temperature is above the boiling point of the ether. The samples were drawn from the artery through a curved glass cannula that reached to the bottom of a test-tube in which there was a small amount of ammonium oxalate powder to prevent clotting. The test-tube was placed in a basin of ice and salt mixture, the blood was run

into the bottom, the tube filled to the top as rapidly as possible, and at once corked. When the blood had cooled almost to the freezing point, the tube was inverted several times for thorough mixing and a sample rapidly pipetted from the bottom and delivered to the bubble tube in the analyzer train as described in a previous paper (1). Duplicate analyses on the same sample invariably gave results within the limit of error stated above.

The analysis of air containing ether vapor was carried out in essentially the same manner (as also described in the previous paper), on 10 to 40 ml. samples. These samples were usually drawn in a mercury burette, measured at room temperature and pressure, and the volume was later calculated to the conditions noted by the methods described in Section 6. The maximum variation in the determination of the ether content of air was 0.002 gm. of ether per liter.

3. Determination of Ether in the Presence of Acetone.

Many hydrocarbon vapors beside ether are oxidized by hot iodine pentoxide, and iodine is liberated. The presence of appreciable amounts of any such substance in the blood or expired air of the animals under study would vitiate the ether determinations. Analysis made of the expired air and blood of the subjects prior to etherization gave no indication of liberation of iodine in excess of the usual blank of the method. The absence of oxidizable substances in the air and blood of normal animals thus indicated does not, however, preclude the development of such volatile substances as the result of the ether anesthesia. It seems reasonable to assume that acetone, either with or without other related substances, is the type of volatile and oxidizable substance which would be found in the blood or expired air. There is some evidence from the literature that such indeed might be the case.

Acetone readily reacts with iodine pentoxide with the liberation of an amount of iodine nearly equivalent to that liberated by a like amount of ether.

Experimentation showed that the passage of the air of the analyzer train through 20 per cent sodium hydroxide heated to 60°C. completely removed amounts of acetone far in excess of any that could be produced in the body. The passage of the air

through the hydroxide does not interfere with the accuracy of the estimation of the ether.

To test the removal of acetone vapor by the hydroxide, a mixture of ether vapor and air was subjected to analysis, with and without the presence of acetone. The ether vapor-air mixture was prepared in a large spirometer by the method described in Section 2. 10 ml. samples of this were analyzed for ether, with

TABLE I.

Showing the Analysis of Ether Vapor in the Presence of Acetone.

An ether vapor-air mixture was made in a large gasometer, and 10 ml. samples were analyzed for their ether content, both with and without passage of the sample through 20 per cent sodium hydroxide solution at 60°C. Acetone vapor to the amount of 5 mg. of acetone was added to other samples and the procedure repeated.

No.	Substance under analysis.	Remarks.	Iodine liberated calculated as ether.
			mg.
1	Ether vapor mixture.	Without passage through sodium hydroxide.	2.12
2	" " "	" " "	2.09
3	" " "	" " "	2.13
4	" " "	Passed through sodium hydroxide.	2.10
5	" " "	" " " "	2.12
6	Ether vapor mixture and acetone vapor.	" " " "	2.15
7	" " "	" " " "	2.16
8	" " "	" " " "	2.16
9	" " "	Without passage through sodium hydroxide.	6.92
10	" " "	" " "	6.41

and without the hydroxide solution, in the analyzer train. The procedure was then repeated after the addition of acetone vapor to the sample. The acetone used was 2 ml. of the vapor drawn from the top of a bottle half full of the substance at room temperature and contained approximately 5 mg. of acetone. The absorption of the acetone was readily demonstrable by adding a few cubic centimeters of Lugol's solution to the hydroxide and noting the odor of iodoform, as in the ordinary test for acetone.

In Table I are given the results from a series of determinations made as described. They show complete removal of the acetone by the hydroxide solution. The use of the hydroxide solution was continued as a routine in all analyses; but in no instance was acetone detected in the blood or expired air of the experimental animals under anesthesia.

Anesthetic ether contains a small amount of water and 2 to 3 per cent of alcohol. Alcohol acts upon the iodine pentoxide in the same manner as ether and, if present in the blood or respired air, would contribute a small additive error to the ether determinations.

In some of the experiments reported, ether was purified by drying over calcium oxide and distilling to half volume. No difference in the ether concentrations of the blood or respired air could be noted when comparison was made between these experiments and those in which unpurified anesthetic ether was used.

4. Laws Governing the Concentration of Ether in the Inspired Air.

When any given weight of ether is evaporated in air, the resultant mixture exerts an increased pressure proportional to the amount of ether present. If expansion is allowed so that the whole assumes the prevailing barometric pressure, a portion of this total pressure, *i.e.*, a partial pressure, is exerted by the ether vapor present. At uniform temperature, this partial pressure is proportional to the weight of ether per unit volume of the air-ether mixture. As shown in the previous paper (1), within the ordinary range of room and body temperatures the vapor follows the laws for a true gas with sufficient conformity for physiological purposes. The weight of ether per liter of air at any given partial pressure can, therefore, be calculated from the common gas laws.

A liter of ether vapor when measured at 0° and 760 mm. pressure is equal in grams to the molecular weight of ether divided by the gas constant, 22.4, and amounts, therefore, to 3.308 gm. The same ether vapor brought to the conditions existing in the lungs, where it is fully saturated with water vapor at 38°, would exert a partial pressure of $B - 49$ mm., in which B is the prevailing barometric pressure and the 49 mm., the water vapor pressure at

38°. The weight of the ether per liter of the mixture would be further decreased through the expansion of the vapor in passing from 0–38°. Thus under lung conditions the weight of 1 liter of ether vapor would equal

$$\text{Weight ether (at 760 and 0°C.)} \times \frac{B - 49}{760} \times \frac{273}{273 + 38} = 2.69 \text{ gm.}$$

At 760 mm. and 38° wet, a liter of ether vapor would exert a partial pressure of 711 mm. and would have a weight of 2.69 gm.

In a calculation of this type, the variation of the prevailing barometer from 760 serves only to limit the maximal partial pressure the ether can exert. This is unimportant in calculating anesthetic tensions since even in the most vigorous inductions the partial pressure of ether in the inspired air never approaches this concentration.

From the gas laws as above applied, both the weight and partial pressure or tension of ether at various temperatures and conditions can be readily calculated.

5. *Distribution of Ether between Blood and Air.*

The coefficient of distribution of ether between air and blood at various temperatures has been worked out in the previous paper (1). It indicates that when an amount of ether is allowed to diffuse between air and blood at 38° until equilibrium is reached, the blood contains 14.9 times as much ether per unit volume as does an equal volume of air. This was later confirmed by the work of Shaffer and Ronzoni (2). This ratio varies inversely with the temperature as is usually the case in solutions of gases or vapors in liquids. At any constant temperature the ether taken up by the blood, when it is exposed to an air and ether vapor mixture, varies in direct proportion to the amount of ether per unit of air and hence to the partial pressure.

When ether is dissolved in blood the blood exerts a tension of ether vapor. The tension so exerted in the blood at any temperature is directly proportional to the amount of ether dissolved; it is equal to the tension or partial pressure exerted in a like volume of air by an amount of ether equal to that in the blood divided by the distribution coefficient at that temperature. Since the solubility of ether in blood varies inversely with the temperature, it fol-

lows that any given amount of ether dissolved in a certain volume of blood exerts a tension which varies directly with the temperature. Thus as the temperature is increased, while the ether concentration is maintained, the tension rises. This is of interest in connection with the observations made by Hans Meyer (3) that with increase in temperature the amount (concentration) of ether necessary to anesthetize some of the lower forms of life is decreased. It at once suggests that the phenomenon of anesthesia depends upon the tension exerted by the dissolved ether rather than upon the actual concentration present. Such, however, is not Meyer's interpretation for he believes the temperature effect to be due to an alteration in the distribution coefficient of the anesthetic between fat and water.

6. Fate of the Ether in the Body.

The data reported below demonstrate that ether is not destroyed or utilized by the body. It is all excreted unchanged. Approximately 90 per cent of ether absorbed can be recovered in the expired air after the cessation of the administration.

The difference in the total amounts of ether in the inspired and expired air during the administration of ether gives the amount absorbed. A continuation of the collection of the expired air after cessation of the ether administration, and the determination of the ether content of the collected air, gives an amount which represents the ether eliminated by way of the expired air. A certain small amount of ether may be eliminated through the skin, in the perspiration of such animals as perspire, and in the urine. The great mass of eliminated ether, however, finds its way into the expired air.

In Table II are given the comparative figures of the total mass of ether absorbed and the amount recovered from the expired air. The average from twelve experiments gives 87 per cent of the absorbed ether eliminated in this manner. The recovery of this amount of ether after administrations which ranged from 21 to 214 minutes duration and the finding of virtually the same percentage recovered irrespective of the length of time excludes any possibility that a portion of the ether is altered or destroyed in the body.

TABLE II.

Showing the Proportion of Total Ether Which is Eliminated in the Expired Air.

Apparatus arranged as described in Section 2. The volume and ether content of inspired and expired air were determined during the period of inhalation of ether vapor. The difference in the ether content between inspired and expired air during the period of ether administration gives the amount of ether absorbed, as shown in Column 3. After cessation of ether administration the expired air was collected until it no longer contained ether and the amount of ether was determined, as given in Column 4. Column 5 shows the percentage of ether recovered.

Experiment No	Duration of ether administration	Ether absorbed.	Ether eliminated in expired air.	Ether eliminated in expired air.
	<i>min.</i>	<i>gm</i>	<i>gm.</i>	<i>per cent</i>
1	120	14.5	13.3	92
2	21	12.2	10.0	82
3	50	12.8	11.1	87
4	15	9.7	7.7	79
5	105	12.8	11.4	89
6	115	9.0	8.2	91
7	91	15.9	13.9	87
8	58	9.0	7.6	84
9	130	20.0	18.4	92
10	214	11.0	9.6	87
11	60	14.6	12.3	84
12	44	20.1	17.7	88
Average, per cent.				87

7. Elimination of Ether Through the Urine and From Exposed Serous Surfaces.

The urine which is secreted during the time that there is ether in the blood reaches an ether concentration approximately equal to that of the blood passing through the kidneys. Like the brain (4), the kidneys have a relatively large blood supply for their bulk and, therefore, little difference in the ether content of the arterial and venous blood. For this reason the urinary content of ether follows the general arterial concentration. This fact has been repeatedly demonstrated during the experimental work by emptying the bladder and analyzing the first few milliliters which collected. Close agreement was invariably found with the arterial blood.

The total amount of ether eliminated by way of the urine is small since the 100 or 200 ml. of the urine secreted during even a long anesthesia could contain no more than 0.15 to 0.30 gm. of ether. The urinary content so found would be representative of the arterial ether concentration during both absorption and elimination in relation to the rate of secretion at the various levels of blood ether. For this reason the urinary ether as determined in a sample voided at the end of etherization gives a composite figure. A sample secreted between catheterizations and during a plateau in the anesthesia can be used to approximate the arterial concentration of ether.

The fact that the urinary ether is the same as that of the blood tends to confirm the fact reported in a previous paper (1), that the solubility of ether in blood and water is practically the same.

The elimination of ether through exposed serous surfaces plays an unimportant part in the general elimination of ether. That such elimination does occur has been demonstrated by Henderson and Haggard (5). In their experiments air incarcerated in the abdominal cavity during anesthesia rapidly assumed an ether partial pressure equal to that in the alveolar air. During this present work this has been repeatedly confirmed.

8. Relation of the Total Amount of Ether Absorbed to the Concentration Breathed.

When a uniform ether concentration is inhaled a point is eventually reached, or closely approximated, beyond which no more ether is absorbed. The body is then saturated with ether at the tension which corresponds to that of the inspired air, corrected for the humidity and temperature existing in the lungs.

The relation between the total amount absorbed and the tension of saturation was determined experimentally for dogs. The animals were allowed to inhale a uniform concentration of ether vapor until no more was taken up, and the inspired and expired airs contained the same mass of ether over any time period. The total amount absorbed was divided by the weight of the animal in kilos to give a basis of comparison with the inspired ether concentration. For the sake of a further datum the ether content of the arterial blood at the time of saturation was also determined. .

In some of the experiments the process of absorption to saturation was expedited by allowing the animals to inhale a relatively high concentration for an initial period; the concentration was then decreased and maintained at the lower level to complete equilibrium.

Table III gives the ether absorbed per kilo and the coefficient of ether distribution between the inspired air and body as a comparison with the blood content. The limited amount of data

TABLE III.

Showing the Total Amount of Ether Absorbed for Various Ether Tensions and a Comparison of the Distribution of Ether between the Inspired Air, the Blood, and the Body as a whole.

Animals were allowed to inhale a mixture of ether vapor in air until no more was absorbed. The total amount taken up was determined from the respired airs. This amount divided by the weight of the animals is compared with the amount in the arterial blood and the concentration in the inspired air.

Experiment No.	Type of animal.	Weight of animal	Concentration of ether in inspired air at 38° wet	Total weight of ether absorbed at saturation.	Ether absorbed per kilo of body weight	Coefficient of distribution between air and body.	Arterial ether content at saturation.
		<i>kg</i>	<i>gm. per liter</i>	<i>gm.</i>	<i>gm</i>	<i>air as unity</i>	<i>gm per liter</i>
1	Old dog, rather fat.	9	0.1	13.0	1.47	1:14.7	1.46
2	Young adult, very lean.	7.5	0.1	9	1.2	1:12.0	1.47
3	Young adult, moderate build	11	0.089	15.9	1.45	1:14.7	1.4
4	Old dog, fat.	17	0.076	20	1.33	1:17.6	1.1
5	Half grown puppy.	9.5	0.089	11	1.16	1:13.1	1.3

presented does not exclude possible exceptions such as might occur in extremely obese individuals. There is in fact, in the figures given, a tendency to somewhat higher coefficients in the older and fatter dogs. The dogs used in this work represented a fair range from lean to moderately obese and from a half grown puppy to a 6 or 8 year old dog. The uniformity of the distribution ratio is sufficient evidence of the consistency of the principle involved.

These experimental findings show that in equilibrium the distribution of ether between the arterial blood and tissues as a whole

may be considered as approximating unity. This does not mean, however, that the arterial blood is an index of body saturation at any time short of complete equilibrium. No such definite relation is maintained.

Although for practical purposes the distribution of ether is fairly uniform throughout the whole body, this is not the exact case, for certain tissues, notably fat, hold more dissolved ether

TABLE IV.

Demonstrating that Absorbed Ether is Distributed Throughout the Body at a Rate Sufficiently Uniform for Practical Consideration.

The numbers in Column 1 indicate the experiment from which the data are taken. The mixed venous blood was drawn from the right heart. The actual weight of ether absorbed was determined from the ether difference in the inspired and expired air up to the time the blood sample was drawn.

Experiment No.	Weight of animal.	Ether content of mixed venous blood	Ether content of arterial blood.	Ether absorbed as calculated from other content of venous blood \times weight of animal.	Ether absorbed by direct measurement.	Variation of calculated.
	kg.	gm. per liter	gm. per liter	gm.	gm.	gm.
1	10	0.5	0.85	5.0	4.2	+0.8
		0.91	1.23	9.1	8.5	+0.6
		1.37	1.51	13.7	14.5	-0.8
2	10	0.7	1.21	7	6	+1.0
		1.4	1.62	14	11.8	+2.2
3	8	0.48	1.21	3.8	4	-0.2
		1.20	1.6	9.6	10.6	-1.0
		1.47	1.71	11.8	12.8	-1.0
4	11	0.61		6.7	5.3	+1.4
		0.92		10.1	9.1	+1.0
		1.1		12	11.2	+0.8

than others; while certain other tissues, the brain and the kidneys, have a more abundant blood supply than others, and for this reason saturate with ether more rapidly. The importance of these exceptions will be discussed in a later section. The fact remains, however, that no tissue or group of tissues reaches full saturation for the tension inhaled until the body as a whole becomes saturated. Nor is the difference in rate of absorption

sufficient to invalidate the general conclusion that the distribution is nearly uniform throughout the body, since only on this ground can the distribution of ether between blood and tissues on a 1 to 1 relation be explained.

If ether is distributed fairly uniformly throughout the body, the mixed venous blood should at any time be an index of the ether content of the body as a whole. To the end of determining the correctness of this view, a number of comparisons have been made between the actual ether content of the body obtained from the respired air and a figure obtained by multiplying the weight of the animal by the ether content of the mixed venous blood. This data is presented in Table IV.

The numbers in the first column indicate the experiment from which the values were obtained some of which are detailed later under the same number. The venous blood was drawn from the right heart by means of a Luer syringe and long needle which was inserted in the lower right thoracic wall of the animal. The arterial blood content, taken at the same time, is also given to emphasize the uncertainty of its content of ether as a measure of that of the body at times previous to saturation.

CONCLUSIONS.

1. The laws governing the concentration of ether in air and its distribution between air and blood are discussed.

2. A modification of the method of ether analysis for use in the presence of acetone is given. In no instance was acetone found in the expired air of animals to which ether was administered.

3. Ether which is absorbed into the body is in no way altered or changed. It is eliminated largely through the expired air. The average of a number of experiments shows that 87 per cent of the total ether absorbed was eliminated through this channel. Ether is also eliminated through the urine, probably also through perspiration, and to some extent from any exposed serous surface.

4. The ether concentration of the urine is virtually that of the arterial blood passing through the kidneys at the moment of secretion. The ether concentration of the urine voided after a period of ether administration is a composite of the various levels of arterial ether and of the varying rate of urinary secretion. The urine

collected between catheterizations and in a plateau of the anesthesia has an ether content equal to that of the arterial blood during the same period.

5. At full saturation with any tension of inhaled ether the distribution of ether between the arterial blood and the tissues as a whole per unit mass is in the ratio of approximately 1 to 1. Therefore, under conditions of full saturation the content in arterial blood multiplied by the body weight is an index of the total ether absorbed. At any period short of equilibrium the arterial blood fails as an index. The mixed venous blood drawn from the right heart serves at all times as an index of the total amount of ether in the body.

6. The conclusions presented in the above paragraph do not preclude the unequal distribution of ether among the various tissues which have different solubilities for ether, nor a more rapid rate of saturation of those tissues which have a relatively large blood supply. It merely indicates a general fact here established experimentally which is not invalidated by minor variations due to unequal distribution. Indeed in the series studied the ratio tended to rise slightly above unity in very obese animals and to fall slightly below unity for young and emaciated animals.

The author wishes to express his indebtedness to Professor Yandell Henderson for valuable criticisms and suggestions on the investigations reported in this series of papers.

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THE ABSORPTION, DISTRIBUTION, AND ELIMINATION OF ETHYL ETHER.

II. ANALYSIS OF THE MECHANISM OF ABSORPTION AND ELIMINATION OF SUCH A GAS OR VAPOR AS ETHYL ETHER.

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The present paper presents a mathematical analysis of the mechanism of ether absorption and elimination. This analysis is fundamental to any conception of the physiology of ether anesthesia. Furthermore, the principles here defined apply in general to any gas or vapor which, like ether, is absorbed and eliminated unchanged; the solubility or coefficient of distribution of each substance determining, according to these principles, the amount and rate of absorption and the relative importance of respiration and the circulation in determining absorption.

Fig. 1 represents diagrammatically the relations of circulation, respiration, and body tissue, which are the factors concerned in the mechanism under study. In this diagram the upper chamber represents the lungs. For convenience the ventilation is considered as a continuous flow of air with the inspired entering at the right and the expired passing out at the left. The partition across the chamber divides the air stream so that a portion does not come in contact with the blood. The area above the partition is the virtual dead space through which the air passes from inspiration into expiration without loss of ether. The remainder of the air, the effective pulmonary ventilation, comes into free diffusion with the pulmonary blood and reaches equilibrium with it.

At the commencement of inhalation the ether taken up by the blood is carried in the arterial stream to the capillaries, where it diffuses into the tissues. The venous blood leaves the tissues with a concentration of ether identical with that of the tissues

through which the blood has passed; and returns to the lungs for a fresh charge. As this continues, the amount of ether, and hence the tension, constantly rises in the tissues. The amount of ether in the venous blood also rises correspondingly and tends

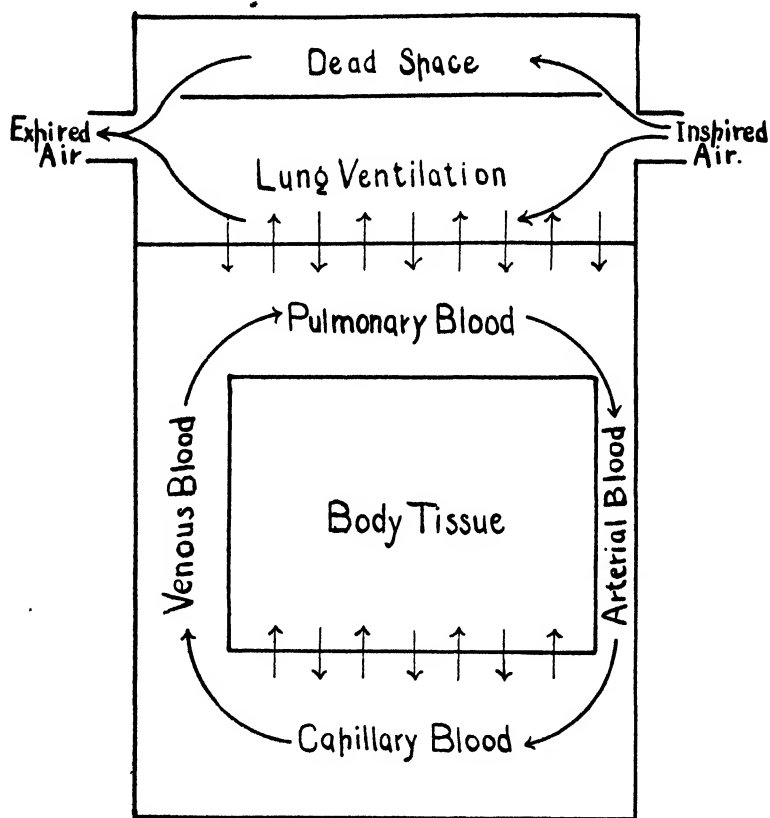


FIG. 1. Diagram illustrating the relations of respiration, circulation, and body tissue: the factor concerned in the absorption and elimination of ether.

to approach that of the arterial. More and more ether is thus carried back to the lungs by the venous blood and a decreasing amount is taken up by the blood from the air in the lungs and by the tissues from the blood. The limit approached is a state of

saturation at which the body will contain an amount of ether equal to the weight of the body multiplied by the concentration in the air breathed, and by the coefficient of solubility (1).

The course of ether absorption is expressed as follows:

If during any time, t , the body becomes saturated to x per cent of full saturation for the tension inhaled, it will in the next time period of the same duration increase in saturation by x

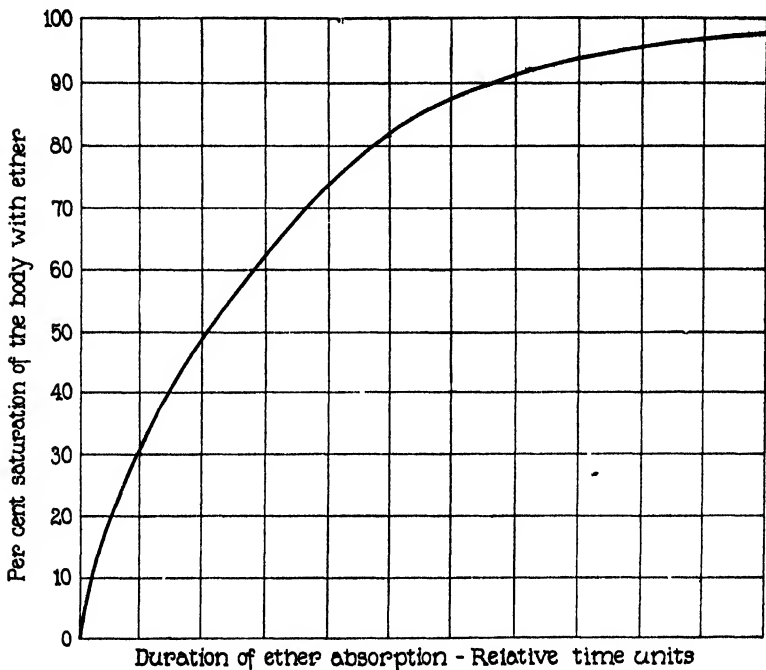


FIG. 2. The theoretical curve of ether absorption.

per cent of the remaining unsaturation. Thus at the end of $2t$, the saturation will be

$$(1) \ x + x(1 - x) \text{ or } 2x - x^2$$

At the end of $3t$, the saturation will be

$$(2) \ x + x(1 - x) + x[1 - (x + x)(1 - x)] \text{ or } 3x - 3x^2 + x^3$$

and so on. This type of mathematical expression gives rise to a curve approaching the logarithmic in shape, as illustrated in Fig. 2.

A similar curve was found by Haldane and coworkers (2) to apply to the absorption of nitrogen.

The ordinate of the curve of Fig. 2 is given in percentage saturation of the body with ether. The abscissa, representing time, has no absolute values, but is a relative scale depending on the conditions under study. To determine the progress of absorption from this curve, the abscissa unit, t , is taken as the point directly below the ordinate value on the curve for the percentage saturation of the body in any arbitrarily chosen time. For each multiple of this value on the abscissa, the percentage saturation of the body is read as the corresponding ordinate value of the curve. Thus an animal which reaches 20 per cent saturation with ether in 10 minutes will be 36 per cent saturated in 20 minutes, and 75 per cent in 1 hour.

The percentage saturation of the body can readily be converted into weight of ether absorbed, or *vice versa*, since the total amount of ether which the body can absorb at any concentration of ether has been defined above.

In order to investigate the influence of variation of blood flow, respiration, and inhaled ether concentration upon the rate of absorption and elimination, it is necessary to analyze the process of absorption more closely. To this end various symbols have been chosen to designate the factors involved.

L , the effective pulmonary ventilation in liters per minute; or volume of breathing minus dead space.

C , concentration of ether in grams per liter of inspired air.

K , distribution coefficient of ether between lung air and pulmonary blood.

It has been determined for ordinary conditions as 15 (3).

W , weight of body in kilos.

G , liters or kilos of blood in active circulation in the body.

B , rate of blood flow through the lungs in liters or kilos per minute.

A_c , arterial concentration of ether in grams per liter of blood.

V_c , venous concentration of ether in grams per liter, for blood drawn from the right heart.

The amount of ether drawn into the lungs during 1 minute of inhalation is the product of the pulmonary ventilation and the concentration of ether in the air, LC . This weight of ether is distributed in the relation of K , (1:15), between the volume of air and the volume of blood passing through the lungs in 1 minute.

It follows from this that each liter of arterial blood leaving the lungs will contain a weight of ether expressed by the equation:

$$(3) \quad A_c = \frac{LCK}{BK + L}$$

The amount of ether absorbed during the first minute of inhalation is the product of the arterial concentration and the blood flow in 1 minute.

$$(4) \quad \text{The weight of ether absorbed in 1 minute} = \frac{LCBK}{BK + L}$$

The amount of ether exhaled from the lungs during this same time is the difference between the amount LC and the value of Equation 4. The amount exhaled can be derived also from a formulation similar to Equation 4.

$$(5) \quad \text{The amount of ether exhaled from the lungs in 1 minute} = \frac{LCK}{BK + L}$$

Throughout the first round of the circulation the absorption of ether is maintained at the rate given by Equation 4. The time consumed in one round of the circulation is obviously that required to move the volume of blood G at the rate of B liters per minute and is therefore $\frac{G}{B}$ minutes. The weight of ether absorbed during the time $\frac{G}{B}$ is expressed by the equation:

$$(6) \quad \text{Grams of ether absorbed during first round of circulation} = \frac{LCGK}{BK + L}$$

The blood is assumed to effect a complete ether equilibrium with the tissues through which it passes. All the data available indicate the general correctness of this assumption. The mixed venous blood from the body, therefore, brings back to the lungs again:

$$(7) \quad \begin{array}{l} \text{Grams of ether brought back to the lungs} \\ \text{during second round of circulation} \end{array} = \frac{LCGK}{BK + K} \times \frac{G}{W}$$

This amount is added to the ether which the breathing brings to the lungs and the sum of the two quantities is distributed

between the arterial blood and the lung air in accord with their volume and at the ratio K , so that the second round of the circulation carries:

$$(8) \text{ Grams of ether absorbed during second round of circulation} = \frac{LCG}{B} + \frac{LCG^2K}{W(BK+L)} \times \frac{BK}{BK+L}$$

The amount given by Equation 8 added to that of Equation 6 represents the total ether absorption at the end of the second round of the circulation; *i.e.*, in time $2\frac{G}{B}$.

The percentage saturation of the body at the end of any time period is obtained by dividing the amount absorbed by the total amount in the body at full saturation for the inhaled concentration; *i.e.*, $C \times K \times W$. The percentage saturation reached in any multiple of time $\frac{G}{B}$ is in the relation expressed by the curve in

Fig. 2, the mathematics of which have been discussed.

The ether content of the arterial blood does not rise gradually from zero as does the general absorption; at the beginning of inhalation its level is suddenly established at a considerable elevation, as shown by Equation 3. From this point the rise continues up to a maximum of $C \times K$ at full saturation. During the first and second round of the circulation the increase is the difference between the value of Equations 6 and 8 divided by the volume of circulating blood, G ; and, thereafter, it follows a course similar, but not exactly parallel, to the percentage saturation of the body as a whole as given in Equations 1 and 2.

The ether content of the venous blood, unlike the arterial, rises gradually from zero, but at full saturation is the same as that of the arterial blood; *i.e.*, $C \times K$ grams per liter. The increase in the ether content of the venous blood has exactly the same expression as the rate of general absorption.

At all times during absorption, equilibrium, and elimination, the ether contents of the alveolar air and of the arterial blood hold the relation defined by the coefficient of distribution. The relation is expressed by the equation:

$$(9) \text{ Ether content of alveolar air in grams per liter} = \frac{Ac}{K}$$

Elimination of Ether Through the Lungs.

The elimination of ether through the lungs follows principles similar to those of the absorption. At the termination of absorption the body contains an amount of ether which is equivalent to full equilibrium with some tension of ether, although this may be much lower than the inhaled concentration with which the body has been brought to partial saturation. If no ether is eliminated, the body forms a closed and static system in which the ether is distributed to the various body constituents and the air in the lungs in proportion to their coefficients of solubility. The concentration of ether in the venous and arterial blood under these conditions is the same. When elimination begins and the air in the lungs is replaced by fresh air, a part of the ether is eliminated in the expired air and the arterial blood leaving the lungs becomes lower in ether concentration than the venous.

The rate at which ether is eliminated through the lungs depends upon the mass of ether in the body, the rate of blood flow, and the volume of air breathed.

The mass of ether brought to the lungs in 1 minute equals the product of the ether concentration in the venous blood, and the volume of blood passing through the lungs per minute; that is, VcB . In one round of the circulation this becomes VcG . The ether brought to the lungs is equilibrated between the air and blood according to the ratio of the coefficient of distribution K . The amount of ether in a volume of air which is the effective pulmonary ventilation for the period is thus eliminated. The amount of ether thus eliminated per minute equals:

$$(10) \quad \frac{LVcBK}{BK + L}$$

In one round of the circulation:

$$(11) \quad \frac{LVcGK}{BK + L}$$

Since the body is 100 per cent saturated for some tension of ether at the beginning of elimination (namely that developed in the pulmonary air under the static equilibrium above discussed), the loss of the same amount of ether expressed by Equation 10

for the first minute or Equation 11, first round of the circulation, will decrease this percentage saturation to an extent expressed by the fraction:

$$(12) \quad \frac{\text{Ether elimination in first period}}{\text{Total ether absorbed}}$$

During the second time period of the same duration an amount of ether will be eliminated equivalent to the same percentage of the ether remaining in the body.

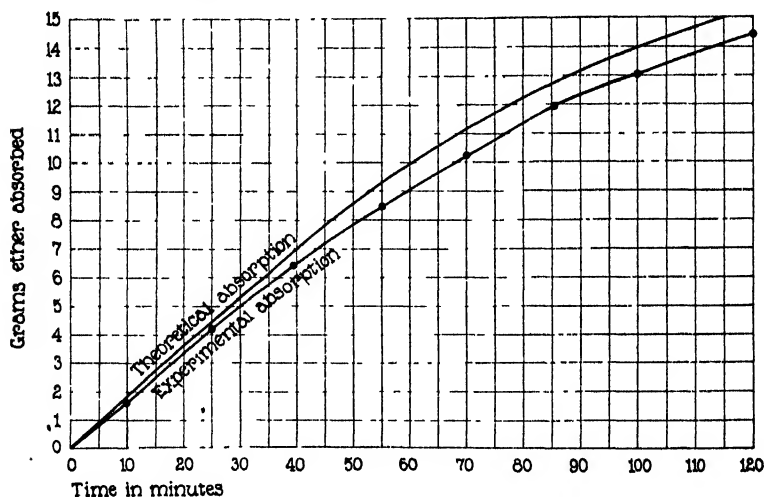


FIG. 3. The curve of ether absorption found in Experiment 1 compared with the theoretical curve calculated by means of the equations given in this paper.

This is obviously the same type of mathematical expression as that discussed for the rate of absorption with Equations 1 and 2, and the curve is the reverse of that given in Fig. 2. Therefore, the elimination is rapid in the beginning and becomes more gradual until the curve is nearly flat, and the final rates of elimination are very slow.

Conformity of Theoretical Rates of Ether Absorption and Elimination to Rates Found Experimentally.

The conception here presented may seem purely theoretical. Its validity is confirmed, however, by the data of such experi-

ments in ether absorption as that presented below. It is one of many closely agreeing experiments. The animal was one in which respiration and blood flow (constant pulse rate) remained throughout the period of absorption practically unchanged, but at a low level because of the heavy morphinization. The procedures and physiological data are presented in Experiment 1. In Fig. 3 these findings are presented graphically as is also the theoretical rate of absorption. The curves actually found are here seen to be in close agreement with the theoretical curves. A number of similar experiments were performed; the details differ slightly, but their main lines conform.

*Experiment 1.**Absorption and Elimination of Ether under Constant Respiration and Circulation.*

Dog, male, weight 10 kilos. Arterial blood drawn from femoral artery. Venous blood from right heart. Connected to inspiratory and expiratory spirometers by trachial tube and valves. During administration of ether, inspiratory air contained 0.20 gm. of ether per liter, measured at 38°, 760 mm. wet. Animal was heavily morphinized 20 minutes prior to etherization. Respiratory volumes were recorded from expired air. Amount of ether absorbed or eliminated was calculated from ether difference in inspired and expired air over measured time.

Time.	Volume of air breathed per min. 20°, 760 mm. wet.	Arterial content of ether.	Venous concentration of ether (right heart).	Pulse rate.	Total ether absorbed or eliminated.	Remarks.
min.	l.	gm. per l.	gm. per l.		gm.	
0	1.57	0.0	0.0	62	0.0	
10	1.51	0.6	0.24	62	1.8	
25	1.60	0.85	0.50	62	4.2	
40	1.50	1.1	0.71	62	6.5	
55	1.50	1.23	0.91	62	8.5	Moderately anesthetized.
70	1.44	1.31		60	10.2	
85	1.35	1.40	1.19	60	12.0	Deeply anesthetized.
100	1.28	1.46		60	13.0	
120	1.28	1.51	1.37	60	14.5	Very deeply anesthetized.

Administration stopped and elimination started.

0						
15	1.8	1.1	1.24	66	1.6	
30	2.7	0.82		74	3.7	
45	2.6	0.57	0.72	74	5.9	
60	3.1	0.37		82	7.4	
75	2.6	0.32	0.41	80	9.5	
90	2.6	0.28		86	10.6	
105	2.9	0.21	0.26	85	11.7	
120	2.6	0.14		85	12.4	
135	2.6	0.09	0.09	90	13.0	
150	2.8	0.07		92	13.3	

The virtual dead space and actual circulatory rate in the animal of this experiment were determined by a slight modification of the general procedure during the 1st minute of the administration. The total air expired during this minute was trapped in a small spirometer and found to be 1.57 liters of air at 20°, 760 mm., wet, containing 0.061 gm. of ether per liter at 38° and 760 mm. wet. In view of the nearly complete absorption of ether from the air of the alveoli, the dead space can be closely estimated, as shown by Henderson and Haggard (4), from the ratio between the inspired and expired ether concentration. In this case the dead space was found to be 30.5 per cent."

The volume of blood flowing through the lungs in 1 minute was determined from the concentration of ether in the arterial blood at the end of the 1st minute. This was found to be 0.32 gm. per liter and as the amount of ether absorbed during the same time was 0.218 gm., the circulatory rate was $\frac{0.218}{0.32} = 0.681$ liters per minute. Although this is a low rate of blood flow for an animal of 10 kilos it is compatible with the slowing of the heart rate (60 to 62 per minute) induced by morphine. The total volume of circulating blood in the animal was assumed to be approximately 1 liter; i.e., 10 per cent of the body weight (5).

The elimination of ether was followed for 2½ hours after the cessation of ether inhalation. The process is somewhat warped by the increased respiration and heart rate coincident with the animal's partial recovery from the morphinization.

Influence of Inspired Ether Concentration upon Rate of Absorption.

The rate at which ether is absorbed under comparable conditions of respiration and circulation is in direct proportion to the concentration inhaled. Under the inhalation of concentrations C and C' , the amounts absorbed during the 1st minute bear the relation:

$$(13) \quad \frac{LCGK}{BK + L} : \frac{LC'GK}{BK + L} \text{ and, therefore, as } C : C'$$

Evidently the relative amounts absorbed under these concentrations during any subsequent minute must also have the relation $C : C'$.

As a result of this proportional rate of absorption it follows that the time required to reach full saturation, or any percentage of saturation, of the body with ether is the same for all ether con-

centrations. The total amount of ether which it is necessary to absorb in order to obtain any degree of saturation of the body varies, like the rate of absorption, in the proportion $C:C'$.

When absorption is considered from the aspect of the comparable time required to absorb a given amount of ether under the inhalation of different concentrations of ether, the simple relations given in the two previous paragraphs are no longer effective. Assume N as the amount of ether which is to be absorbed under inhalations of concentrations C and C' . At full saturation with C and C' the body would contain amounts of ether CKW and $C'KW$, respectively. After the absorption of the amount N , the body will in each case be $\frac{N}{CKW}$ and $\frac{N}{C'KW}$ per cent saturated.

These factors stand to each other in the ratio $\frac{1}{C} : \frac{1}{C'}$, but the respective times differ widely from this ratio, for we know from the curve of Fig. 2 that the rate in percentage of saturation at which the body absorbs ether under the inhalation of any uniform concentration of the vapor is rapid during the early periods and slow later. Increase in the concentration inhaled results, therefore, in a much more than proportional decrease in the time required to absorb any given amount of ether.

To illustrate this point, let us assume that C and C' bear the simple relation of 1 to 2, and that the amount N corresponds to 90 per cent saturation at concentration C . The times required to absorb the amount N will then bear the same relation to each other as the abscissa values of 45 and 90 per cent saturation on the curve of Fig. 2. These bear the relation of 1 to 4.4, thus showing the disproportionately rapid increase of absorption following increase of inhaled ether concentration.

These relations are illustrated by a comparison of Experiment 2 with Experiment 1; the two experiments being closely similar except that in one the ether concentration inhaled was 0.5 gm. per liter and in the other 0.2 gm. The conditions and quantities as regards circulation, respiration, and body weight, were essentially the same in both. The blood flow through the lungs in Experiment 2 was 910 cc. per minute and the dead space 34.2 per cent. The volume of respiration was reduced to 2.1 liters a minute by morphinization.

Although the ether concentration in Experiment 2 was only two and a half times as great as that in Experiment 1, full anesthesia resulted in 10 minutes, as compared to 55 minutes in the first experiment, and failure of respiration, as a result of the high concentration of ether in the blood, occurred in 21 minutes. After 4 minutes of manual artificial respiration, spontaneous breathing was resumed. The course of elimination of ether did not vary greatly from that of Experiment 1.

Experiment 2.

Showing the Absorption and Elimination of Ether under Constant Volume of Respiration and Circulation and with a High Concentration of Ether in the Inspired Air.

Dog, male, weight 10 kilos. Procedure similar to that of Experiment 1. Animal breathed ether concentration of 0.5 gm. per liter. Preliminary morphinization.

Time.	Volume of air breathed per min. 20° 760 mm. wet.	Arterial content of ether.	Venous concentration of ether (right heart).	Pulse rate.	Total ether absorbed and eliminated.	Remarks.
min.	l.	gm. per l.	gm. per l.		gm.	
0	2.1	0.0	0.0	71	0.0	Ether started.
10	2.2	1.4	0.9	71	6.0	Fully anesthetized.
20	1.8	1.62	1.4		10.4	Very deeply anesthetized.
21					12.2	Breathing stopped.
						Artificial respiration started.
25						Spontaneous breathing re-established.

Ether stopped and elimination started.

0						
10	1.8	1.1	1.2			
20	2.6	0.75	0.81		3.4	Some movement of limbs.
60		0.41	0.49		6.5	Fully conscious.
120	2.8	0.2	0.26		9.4	
160		0.02			10.0	

Influence of Volume of Respiration upon Rate of Ether Absorption and Elimination.

Under inhalation of a uniform concentration of ether vapor, the rate of absorption varies nearly, but not quite, in exact proportion to the volume of air breathed. Under the 2 volumes of respiration L and L' the respective rates of absorption bear the relation:

$$(14) \quad BK + L' BK + L'$$

The slight disproportionality is due to the fact that the variable amount of air shares its ether content with a constant volume of blood.

The time, t , required to absorb a given amount of ether or reach the same percentage saturation of the body under different volumes of breathing is shown by the ratio:

$$(15) \quad t : t' :: L' (BK + L) : L (BK + L')$$

To illustrate this relation by a practical example, let us assume that an animal with a blood flow, B , of 2 liters per minute, absorbs 8 gm. of ether in 12 minutes. The same animal at double the volume of breathing will absorb the same amount of ether in 6.4 minutes. A 50 per cent reduction in the volume of pulmonary ventilation would result in a time of 22.2 minutes to absorb the 8 gm. of ether.

The rate at which ether is eliminated from the body is just short of proportional to the volume of pulmonary ventilation. The rates under the volumes L and L' are in the relation:

$$(16) \quad \overline{BK + L' BK + L'}$$

Augmentation of breathing offers practically the only means by which the elimination of ether from the body can be expedited. Advantage has been taken of this fact by Henderson, Haggard, and Coburn (5) and by White (6). In order to induce rapid deetherization after surgical operations, thus greatly decreasing nausea and vomiting and conserving the vitality of the patient,

they have augmented the volume of breathing by means of carbon dioxide inhalation.

By the use of oxygen plus 5 per cent carbon dioxide, Henderson and Haggard (7, 8) have found that the elimination of carbon monoxide may be similarly accelerated.

Although, as pointed out above, the absorption and elimination of ether vary practically proportionately with the volume of lung ventilation, this is not the case with less soluble gases. The lower the value of K , the distribution coefficient of the gas between blood and air, the less is the influence of the volume of air breathed upon the rate of absorption. The absorption or elimination of an insoluble gas, such as hydrogen or nitrogen, is very slightly affected by variation in respiration, but is nearly proportional to the volume of the blood flow through the lungs. With such a gas as ethylene, also, the blood flow is a more important factor than the respiration.

In all the foregoing discussions the term "effective lung ventilation" has been used in distinction to volume of respiration. The relation of the two is expressed by the equation:

(17) Volume of pulmonary ventilation per minute (L) = Volume air breathed minus virtual dead space \times rate of breathing.

As the virtual dead space had been shown by Henderson and Haggard (4) by means of ether to be approximately one-third of the tidal air, it is practically correct to set down:

(18) Volume of pulmonary ventilation = Volume of air breathed $\times \frac{2}{3}$.

Experiment 3 indicates the course of the absorption of ether in an animal where breathing was increased to four times the resting volume by means of inhalation of 7.5 per cent of carbon dioxide. After the cessation of ether administration the inhalation of carbon dioxide was continued to expedite the elimination of ether.

Experiment 3.

Showing the Rapid Induction of Anesthesia and Subsequently the Accelerated Elimination of Ether by the Augmentation of Respiration under Inhalation of CO_2 .

Dog, male, weight 9 kilos. No preliminary morphinization. Animal breathed a concentration of 0.2 gm. of ether per liter of air. General

procedure similar to previous experiments except that all inspired air contained 7.5 per cent CO_2 .

Time.	Volume of air breathed per min. 20°, 760 mm. wet.	Arterial ether concentration.	Amount absorbed and eliminated.	Remarks.
min.	l.	gm. per l.	gm.	
0	3.2	0.0	0.0	Inhalation of 7.5 per cent CO_2 begun.
5	12.8	0.0	0.0	
0		0.0	0.0	Inhalation of ether started.
2		1.2	3.1	Moderate anesthesia.
10	12.0	1.46	9.7	Deep anesthesia. Ether stopped.

Elimination started.

0				
3	12.4			Fully conscious and apparently normal.
10	13.6	0.3	5.6	CO_2 stopped.
20	4.2	0.1	7.4	
30	4.4		7.7	

Influence of Rate of Blood Flow on Absorption and Elimination of Ether.

The rate of blood flow has very little influence upon the rate at which ether is absorbed. Thus, under the conditions of constant volume of respiration and constant concentration of inspired ether and with the rates of blood flow B and B' , the rates of absorption for the 1st minute will vary as:

$$(19) \quad \frac{B}{BK + L} \frac{B'}{B'K + L}$$

With the high distribution coefficient K , for ether the variation in this ratio is very slight. For example, assume the rates B and B' as 2 and 4 liters per minute, respectively, and L in each case as 2 liters of lung ventilation per minute, the ratio resolves

into the values; 6.25:6.45. Under conditions here assumed, a 100 per cent increase in the blood flow results in only 3.2 per cent increase in the rate of absorption.

The rate of blood flow plays a comparatively unimportant part also in determining the rate at which ether is eliminated. It is true that with similar concentrations of ether in the venous blood the amount of ether brought to the lungs is proportional to the volume of blood flow. The total of ether thus brought in any period of time is equilibrated between the same amount of air and larger or smaller amounts of blood containing ether. The slight change in the elimination of ether induced by alteration of blood flow from B to B' is expressed by the ratio given in Equation 19.

CONCLUSIONS.

1. A mathematical analysis of the mechanism of ether absorption and elimination is presented. The principles here defined are applicable also to any gas or vapor which like ether is absorbed and eliminated unchanged.

2. The rate at which ether is absorbed under comparable conditions of respiration and circulation is in direct proportion to the concentration inhaled. The time required to reach full saturation or any given percentage of saturation of the body is the same for all ether concentrations. On the other hand, the time required to absorb any given amount or mass of ether under inhalation of different concentrations is disproportionately rapid for the higher concentrations.

3. The rate at which ether is absorbed or eliminated varies nearly, but not quite, in exact proportion to the volume of air breathed. The rate of blood flow has very little influence upon the rate of ether absorption or elimination. The relatively great influence exerted by the volume of air breathed is occasioned by the high solubility coefficient of ether. The reverse relation exists with comparatively insoluble gases.

4. A full experimental foundation for the use of CO_2 in connection with rapid induction and elimination of ether is laid.

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THE ABSORPTION, DISTRIBUTION, AND ELIMINATION OF ETHYL ETHER.

III. THE RELATION OF THE CONCENTRATION OF ETHER, OR ANY SIMILAR VOLATILE SUBSTANCE, IN THE CENTRAL NERVOUS SYSTEM TO THE CONCENTRATION IN THE ARTERIAL BLOOD, AND THE BUFFER ACTION OF THE BODY.

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In the two previous papers of this series (1, 2) a general demonstration has been made of the fact that ether is distributed throughout the body as a whole in approximately the same concentration that it exists in the blood. At the same time it was indicated that this cannot apply to any one tissue or organ but is an expression of the integration of all the tissues; some of which take up more and some less ether than others.

The present paper deals with the absorption of ether by the central nervous system and the part which the peculiarities of this absorption play in the development of anesthesia.

Anesthesia as a Result of the Action of Ether upon the Brain.

In respect to the anesthetic action of ether, the body may be divided into two classes of tissues; those upon which ether has an action resulting in the phenomena of anesthesia; and those upon which ether has no physiological reaction of this sort. This division at once places the nervous system in the first class and the remainder of the body in the second.

The general phenomena of anesthesia, loss of consciousness, and reduction or abolition of the reactions to pain, are due solely to the action of ether upon the brain. As evidence that such is the case, the following experiment is significant. A 5 per cent solution of ether in saline solution was injected under pressure

from a burette through a hypodermic needle into the carotid artery of a 10 kilo dog. The rate of flow was adjusted to feed approximately 0.5 gm. of ether per minute into the uninterrupted and otherwise unaltered blood stream to the head. During the administration the animal presented all the indications of general anesthesia. Immediately after the completion of the injection the venous return from the head contained 0.9 gm. of ether per liter. Arterial blood drawn from the femoral artery at the same time contained only 0.03 gm. of ether per liter.

From this experiment it is evident that the concentration reached in the central nervous system is the determining factor in the anesthetic action of whatever amount of ether is at the time in the body. The central nervous system constitutes in the adult about 5 per cent of the total body weight. The ether in the body is thus distributed between a small bulk upon which it has physiological action and a far larger bulk upon which, for our purpose, it has virtually none. Unfortunately, during administration of ether by inhalation the larger bulk exerts an almost controlling influence over the rate at which an anesthetic concentration can be built up. It acts through its relatively great mass as an enormous buffer.

Factors Governing the Rate at Which Ether Accumulates in the Brain.

The maximum amount of ether which any organ or tissue can absorb at a given tension is determined by the solubility of ether in that tissue. The rate at which ether accumulates in any tissue depends both upon the solubility of ether in that tissue and also upon the amount of ether brought to the tissue in any period of time. The latter factor is determined by the blood supply in relation to the bulk of the tissue. Thus, a tissue with a comparatively low solubility for ether and a large blood flow approaches saturation rapidly, while a tissue of high solubility and small blood supply approaches saturation much more slowly.

The blood supply to the brain is relatively very large as compared with the rest of the body. In regard to the solubility of ether in the tissue of the central nervous system the literature is somewhat contradictory. The figures given by Nicloux (3) for the distribution of ether in various organs of the body at full

anesthesia indicate that the nervous tissue contains an amount quite close to that in an equal volume of the arterial blood. On the other hand, Frantz (4) found under the same conditions that the brain contained nearly twice as much as the blood.

To throw further light on this matter several experiments along the following lines were performed. A weighed quantity of finely mascerated brain tissue from a dog was placed in a flask which was then filled with a measured amount of blood plasma or saline solution containing a known weight of ether. The flask was stoppered, and the mixture was agitated in a water bath at body temperature for several hours. At intervals the agitation was stopped long enough for the particles of tissue to settle out of the liquid; small samples of the liquid were then drawn and analyzed for their ether content. When no further drop in ether concentration of the liquid occurred, the solubility of ether in the nervous tissue was calculated from the respective weight of fluid and tissue and from the initial and final concentration of the plasma or saline solution. The results of five such experiments gave an average of 1.14 as the solubility of ether in nervous tissue when compared with blood as 1. The extremes found were 0.92 and 1.31. The method is not sufficiently accurate to warrant more than the general statement that nervous tissue has for all practical purposes about the same solubility coefficient for ether as has blood. This does not imply, however, that certain of the constituents of brain tissue may or may not take up more or less ether than others under the same conditions. It suffices here that the distribution of ether between blood and brain tissue is approximately unity.

With the solubility of ether in the nervous tissues thus indicated and with the relatively large blood supply of the central nervous system, it is evident that the brain and spinal cord will approach saturation at the tension in the arterial blood at a much more rapid rate than the rest of the body. In this connection it may be pointed out that all brief surgical operations done under ether, are performed while only the nervous system contains an anesthetic concentration, but the body much less: a very desirable condition.

*Relation of Ether Content of Arterial Blood and of Blood
from the Internal Jugular Vein.*

The ether content of the blood drawn from the unobstructed internal jugular vein is a direct measure of the ether content of the brain. This conclusion, which follows from the discussion presented in Paper II, is further substantiated by the following observations: (a) A number of ether determinations made upon the cerebrospinal fluid drawn during the course of ether administration gave ether contents in very close agreement with those of the blood from the internal jugular vein. (b) The physiological condition of the dog during the inhalation of ether was closely paralleled by the concentration of ether existing in the blood from the internal jugular vein.

During the inhalation of ether, and especially at first, the ether content of the blood drawn from the internal jugular vein approaches more quickly and more nearly to the arterial than does that of the general venous blood from the right heart.

Similarly, at the beginning of elimination, the ether content of blood from the internal jugular vein continues for some time at a higher level than either the arterial or general venous blood. This relatively high ether content of the jugular blood results from the fact that during absorption, especially when brief, the brain is much more nearly saturated with ether than is the rest of the body. At the beginning of elimination the general arterial blood falls slightly below the ether content of the general venous blood, for under the usual volume of breathing only a small percentage of the ether in the blood is eliminated during each passage through the lungs. The relatively high level of ether in the internal jugular blood is, however, not long maintained; for as elimination progresses the deetherization of the brain is more rapid than is that of the body as a whole, just as its absorption was more rapid, because of its large blood supply. The ether content of the blood from the internal jugular vein, therefore, quickly falls a little below that of the general venous blood, but remains necessarily slightly above that of the arterial blood.

These relations are illustrated by Experiments 1, 2, and 3, carried out in essentially the same manner as were the experiments on absorption given in the previous paper. Blood samples

were drawn practically simultaneously from the femoral artery, internal jugular vein, and the right heart, and their ether content was determined.

Experiment 1.

Showing Relation between the Ether Content of the Arterial, Internal Jugular, and Mixed Venous Bloods.

Dog, male, weight 14 kilos. General procedure as in experiments described in previous paper (2). Animal breathed air containing 0.3 gm. of ether per liter 38°, 760 mm.

Time.	Ether content of blood.			Remarks.
	Arterial.	Mixed venous.	Internal jugular.	
<i>min.</i>	<i>gm. per l.</i>	<i>gm. per l.</i>	<i>gm. per l.</i>	Ether started.
0	0	0	0	
2	0.46	0 09	0.18	
10	0.84	0.46	0.67	
20	1.15	0.78	1.09	
30	1.41	1.12	1.38	
Administration of ether stopped and elimination started.				
2	0.96	1.00	1.26	.
10	0.48	0.57	0.52	
30	0.18	0.22	0.19	

Experiment 2.

Showing Relation between the Ether Content of the Arterial, Internal Jugular, and Mixed Venous Bloods.

Dog, male, weight 10 kilos. Procedure as in previous experiment. Animal breathing air containing 0.5 gm. of ether per liter (38°, 760 mm.).

Time.	Ether content of blood.			Remarks
	Arterial.	Mixed venous.	Internal jugular.	
<i>min.</i>	<i>gm. per l.</i>	<i>gm. per l.</i>	<i>gm. per l.</i>	Ether started.
0	0	0	0	
10	1.40	0.90	1.25	
20	1.62	1.41	1.51	
Administration of ether stopped and elimination started.				
10	0.75	0.81	0.78	
60	0.41	0.49	0.43	

Experiment 3.

Showing Relation between the Ether Content of the Arterial, Internal Jugular, and Mixed Venous Bloods.

Dog, male, weight 10 kilos. Procedure as in previous experiments. Animal breathing air containing 0.20 gm. of ether per liter.

Time.	Ether content of blood.			Remarks.
	Arterial.	Mixed venous.	Internal jugular.	
<i>min.</i>	<i>gm. per l.</i>	<i>gm. per l.</i>	<i>gm. per l.</i>	Ether started.
0	0	0	0	
10	0.6	0.24	0.46	
40	1.1	0.71	0.91	
85	1.40	1.19	1.26	
120	1.51	1.37	1.43	
Administration of ether stopped and elimination started.				
15	1.1	1.24	1.16	
75	0.32	0.41	0.36	
120	0.21	0.14	0.22	

The practical conclusion to be drawn from this evidence is that the initial level of ether reached by the arterial blood is of the utmost importance in determining the rapidity of induction of anesthesia. The absorption of a relatively small mass of ether under conditions which tend to produce a high concentration in the arterial blood results in a rapid induction, while the absorption of a large amount of ether slowly brings the buffer action of the body as a whole into action and results in a prolongation of the early stages of anesthesia.

Factors Influencing Amount of Ether in Arterial Blood.

Under uniform respiration and circulation the ether concentration in the arterial blood varies directly with the concentration of ether inhaled. The mathematical expression of the concentration of ether in the arterial blood during the 1st minute of absorption, as given in Paper II, is

$$(1) \frac{LCK}{BK + L}$$

in which L is true breathing, C concentration of ether inspired, B blood flow through the lungs, and K the distribution coefficient of ether between air and blood in the lungs. Evidently the quantity indicated by this expression varies in proportion to C .

During the inhalation of a constant concentration of ether vapor it is possible, however, to have wide fluctuations in the arterial content of ether as a result of variations in the volume of air breathed. In fact with increase or decrease of the volume of breathing the amount of ether in the arterial blood is very nearly, though not quite completely, proportional. The relation for the respective amount of ether in the arterial blood, A_c and A'_c under the conditions of respiration L and L' , all other conditions being the same, is:

$$(2) A_c:A'_c::\frac{L}{BK+L}:\frac{L'}{BK+L'}$$

Thus, for example, if we assume the case of an animal with a blood flow of 2 liters per minute, inhaling into the lungs 4 liters per minute of air containing 0.4 gm. of ether per liter, the initial level of ether in the arterial blood will be 0.7 gm. per liter. On doubling the ventilation, while the other factors remain constant, the ether content of the arterial blood will become 1.26 gm. per liter. On the other hand, if respiration be decreased to 50 per cent of the original the arterial ether will fall to 0.37 gm. per liter.

The influence of volume of respiration is greatest at the beginning of the inhalation and is diminished progressively as the inhalation is continued. This diminution is due to the fact that the increasing amounts of ether returning in the venous blood tend to decrease the further absorption and to stabilize conditions.

The rate of blood flow does not greatly influence the rate of absorption, although its effect upon the initial level of ether reached in the arterial blood is very marked. Thus under conditions of uniform ether inhalation and respiration and with the two rates of blood flow B and B' the respective levels of ether in the arterial blood will bear the relation:

$$(3) \frac{B}{BK + L} \cdot \frac{B'}{B'K + L}$$

Therefore, halving the rate of blood flow results in nearly doubling the initial arterial level of ether, while an increase in the rate of blood flow is followed by a nearly proportional drop in the level. The amounts absorbed, however, differ only slightly.

At the moment of cessation of the administration of ether the arterial blood falls at once to a little below the ether content of the returning venous blood; and during the elimination of ether through the expired air the arterial content is diminished below that of the venous by an amount which is determined by the rate of elimination. In any period of time the venous blood brings to the lungs an amount of ether determined by the venous concentration and the volume of blood returned to the lungs, or $V_c \times B$. From this mass of ether a certain amount (with the ordinary volume of breathing, only a small percentage) is eliminated in the expired air during each round of the circulation. The remaining ether is left in the arterial blood and passes on to the tissues to be there again equilibrated with the slightly higher average concentration which they contain; and then back to the lungs again. The relation of the arterial concentration, A_c , to the venous is shown by the expression:

$$(4) A_c = \frac{V_c B - \text{ether eliminated in 1 minute}}{B}$$

From this expression it is evident that those factors which influence the rate of elimination also determine the difference in ether content of the venous and arterial blood. It is also to be kept in mind that the return of consciousness is dependent on the lowering of the arterial concentration. The greatest factor, therefore, in widening the difference between the ether contents of the venous and arterial blood and restoring consciousness is the volume of pulmonary ventilation.

Two Types of Ether Apnea.

The close correlation between the degree of brain saturation, the ether content of the arterial blood, and the factors which

control the latter, affords an explanation of some of the phenomena commonly observed, but inadequately explained, during the administration of ether, and during recovery.

In the forced induction of anesthesia high concentrations of ether are often employed. The immediate result is a sudden rise of the concentration in the central nervous system to a level sufficient sometimes to cause cessation of breathing. As no large amount of ether has been absorbed into the body, however, and the cessation of breathing stops any further immediate absorption, the ether in the arterial blood falls in a few seconds to the low level of the mixed venous blood. The brain is thus quickly relieved of its excess of ether by the blood flowing through it and breathing usually soon reestablishes itself spontaneously. This is one type of ether apnea.

There is also another and more serious type of respiratory failure which does not so readily clear up. It occurs as the result of a high ether concentration in the inspired air after the body has absorbed a considerable amount of this substance. Under these circumstances the mixed venous blood is also at a fairly high level of ether, and with the cessation of breathing the concentration in the brain is not so rapidly reduced by the blood. It then usually becomes necessary to institute artificial respiration in order to maintain the supply of oxygen until sufficient ether has been shifted by the blood through the brain to allow the resumption of spontaneous breathing.

These alterations of breathing and apnea are produced most intensely during inapt induction by the method in which a cone is employed. The ether is poured over a porous membrane through which both the inspired and expired air passes, while the ether is volatilized. It is practically impossible to maintain even an approximately constant amount of ether in the inspired air by this method. When the volume of respiration is fluctuating widely, the period of induction is unduly prolonged, with alternate periods of excessive breathing, during which the subject returns to partial consciousness, and periods of apnea due both to ether and to overbreathing and temporary reduction of the carbon dioxide in the arterial blood and respiratory center.

Even a few fairly strong whiffs of ether have an analgesic action, although the total amount of ether absorbed would be insuffi-

cient, if distributed evenly over the body, to produce any noticeable physiological effect. During the brief inhalation, however, the arterial blood rises to a fair degree of ether content, and the brain is correspondingly affected because of its large blood supply.

During the elimination of ether and recovery from anesthesia it is often observed that the subject returns temporarily to consciousness if a period of hyperpnea occurs. The increased ventilation causes a widening of the difference between the venous and arterial levels of ether and thus decreases that of the brain. With the relapse of respiration to the normal or a subnormal volume the subject passes once more into unconsciousness, for the arterial level then rises again more nearly to that of the venous. This phenomenon is particularly noticeable following the rapid elimination of ether by means of carbon dioxide inhalation (5).

From the evidence presented in this and the preceding papers it is clear that the ideal ether induction and anesthesia is one in which the ether concentration of the blood is maintained at a full anesthetic level from the beginning. But to do this safely the concentration of ether inhaled must be one which will not allow the arterial content to rise to the point of respiratory failure nor cause lung irritation. This topic is elaborated in the following paper.

CONCLUSIONS.

1. It is here shown experimentally that the ether concentration reached in the central nervous system, not that in the body as a whole, is the determining factor in the anesthetic action of ether.
2. The rate at which any tissue approaches saturation with ether at the tension in the arterial blood is in an inverse relation to its solubility coefficient and in direct proportion to its volume of blood flow.
3. The central nervous system has a much larger blood flow than have other tissues, as shown by the rapidity with which the concentration of ether in the blood of the internal jugular vein approximates the arterial concentration. The solubility coefficient of brain tissue for ether is approximately the same as that of the blood.

4. When ether is absorbed it is distributed between a small bulk upon which it has a physiological action, the central nervous system, and a large bulk, the rest of the body, upon which it has virtually no action. The body thus acts through its large capacity for ether as an enormous buffer to delay both induction of anesthesia, and later the elimination of the anesthetic.

5. Because of its large blood supply, the brain rapidly approaches saturation at the tension of ether in the arterial blood. The ether content of the blood from the internal jugular is an index of brain saturation and runs only a little below that of the arterial blood. The mixed venous blood of the right heart is an index of general body saturation. It approaches the arterial level very slowly. It is thus that the buffer above referred to, retards the induction of anesthesia.

6. The ether content of the arterial blood is the critical factor in determining the degree of anesthesia at the time. This factor bears no immediate relation to the total amount of ether which has been absorbed into the body.

7. The level of ether in the arterial blood is altered in proportion to change in concentration of ether inspired; the respiratory volume exerts a control just short of proportionality; while rate of blood flow influences the arterial concentration inversely.

8. During elimination of ether the concentration in the brain is only slightly higher than the arterial concentration. At the beginning of elimination the jugular blood may continue for a time at a higher level than the general venous blood. As the elimination progresses the difference in the level of arterial and general venous blood is determined by the volume of respiration and rate of blood flow. The volume of breathing is by far the predominating factor.

9. The different types of apnea under ether are explained.

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THE ABSORPTION, DISTRIBUTION, AND ELIMINATION OF ETHYL ETHER.

IV. THE ANESTHETIC TENSION OF ETHER AND THE PHYSIO- LOGICAL RESPONSE TO VARIOUS CONCENTRATIONS.

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In a recent paper by Ronzoni (1), which appeared in print after the present work had been completed, there is presented a summary of the anesthetic tensions of ether found by previous investigators. The very extensive work of Gramen (2) is not included in this review nor are the recent findings of White (3). Although the preponderance of evidence points to a concentration of 3 to 3.5 per cent by volume of ether vapor as the anesthetic tension, there exists an apparent incongruity which is set forth here briefly and which makes the present author, for reasons which will be apparent, hesitate to dogmatize.

The blood of anesthetized subjects, man or animal, has been found by most investigators (1, 2, 3, 4) to contain from 1.0 to 1.5 or 1.7 gm. of ether per liter. The extremes express light and deep anesthesia. Upon these values there is complete experimental agreement and further confirmation in the present work. In a previous paper the writer has determined the ratio of distribution of ether between air and blood as 1 to 15; and Shaffer and Ronzoni (5), employing an entirely different technique, have confirmed this ratio. When this coefficient is applied to the accepted concentration of ether in the blood at anesthesia, the corresponding tension of ether vapor in the respired air becomes 2.6 to 4.0 per cent by volume.

Spencer (6) and Ronzoni (1) have kept animals anesthetized for long periods through the inhalation of air containing from 3 to 4 per cent of ether vapor. In the present work a similar result will be reported. In all these investigations the concen-

tration was determined by analysis; Spenzer employed combustion, Ronzoni a modification of the Nicloux method; while the writer has used the iodine pentoxide method described in an earlier paper (7). In addition Waller (8), using a gas balance (the method later employed by Boothby), found an approximate value of 4.11 per cent for the anesthetic tension; while Dresser (9), who analyzed the air from a Julliard mask during anesthesia, found it to contain approximately 3.5 per cent of ether vapor.

In contrast to all the foregoing work, Boothby (10), using a Connell anesthetometer (11), recalibrated by means of a Waller balance (12), maintained a surgical patient in good anesthesia while breathing, in some instances for several hours, an approximately 7 per cent mixture of ether vapor and air. This work has been widely accepted, and the value of 7 per cent as the anesthetic concentration of ether has found its way into numerous text-books.

The preponderance of evidence is overwhelming against the value given by Boothby, and yet in the opinion of the author it cannot be dismissed upon the mere assumption of error or faulty technique. There exists in the situation an incongruity which is suggestive of something more than mere error. In this connection the work of Spenzer, quoted above, is of particular interest. In order to determine the percentage of ether vapor necessary to maintain anesthesia in dogs and cats, he volatilized a carefully weighed mass of ether in a measured volume of air. According to calculation the mixture should have contained 6.7 per cent of ether vapor; on analysis it was found to contain one-half this amount or 3.4 per cent. Spenzer placed faith in his analytical findings, while Boothby, for obvious reasons, preferred the calculated value. The anesthetic mixture used by Boothby was prepared in a manner rather similar to that of Spenzer and analyzed, as stated, by a physical rather than a chemical method.

In carrying out the work presented in this series of papers, the author has been repeatedly confronted by a situation similar to Spenzer's. Carefully prepared ether-air mixtures have given an analysis of almost exactly one-half the calculated figure.

A prolonged and unsuccessful effort has been made to account for the discrepancy in the results. The question of ether loss has been considered from every aspect. Thus the concentration of the ether-air mixture contained in the gasometer has been held

for many hours without appreciable drop. The discrepancies between the calculated and analytical results were never of an order of magnitude other than the ratio of approximately 2 to 1; the phenomena showed no regularity. The very doubtful possibility of a polymerization of ether was considered, but without result.

As a final check upon the analytical findings a sample of the mixture used for inducing and maintaining anesthesia was dried and passed through a condenser surrounded with liquid air. The ether thus recovered had the ordinary characteristics of ether including the vapor pressure. Furthermore, the amount recovered was in agreement with the results obtained by the iodine pentoxide analysis. Upon this basis the incongruity was allowed to rest and the analytical figures accepted at face value.

On this basis the anesthetic tension of ether vapor is 3.0 to 4.0 per cent. But why very close to one-half of the ether vapor volatilized is so frequently lost remains unexplained.

Determination of the Anesthetic Tension of Ether.

Only at full equilibrium for any tension of ether inhaled can the physiological state be correctly correlated with the inspired or expired ether concentration, or with that of the blood drawn from any source. Even urine secreted at this time may serve as a valid index.

To determine the state of equilibrium during the course of ether inhalation it is necessary that the inspired and expired airs, collected over any period, shall contain exactly the same mass of ether; thus showing that absorption no longer takes place. In passing it is to be observed that the word "mass" is used advisedly here, since the respective concentrations may be dissimilar as a result of difference in volume of the inspired and expired air consequent upon the character of the subject's respiratory quotient.

Equilibrium can also be determined by the agreement of the ether concentration of the arterial blood and that drawn from the right heart. Emphasis must be placed upon the point that blood drawn from any other source than the right heart is in no way indicative of the degree of saturation of the body with ether. The assumption that the venous blood, drawn for example from the femoral or the median basilic vein, is representative of the ether

content of the general venous blood is a very common error. The concentration of the venous blood from any particular organ indicates the concentration of ether and the degree of approach to equilibrium in the particular structure from which the blood is collected, but does not indicate a similar state in other organs or in the body as a whole.

During the elimination of ether, especially if the breathing is moderate, the ether contents of the general venous and arterial bloods commonly differ by only a slight margin so that during this time the ether content of the blood drawn from any source is a fair index of the degree of saturation of the body as a whole. White (3) has taken advantage of this fact in estimating the physiological state at various ether concentrations in the blood during recovery from anesthesia.

The method of determining the physiological response to ether through the correlation of the condition at equilibrium with some inhaled tension of ether cannot readily be applied to a state less than that of full anesthesia. The bodily activities of a lesser degree of anesthesia, particularly the augmented respiration, rapidly lead to alterations in the subject's condition quite aside from those induced directly by the anesthesia. On the other hand, the continued inhalation of a concentration sufficient to induce full anesthesia is accompanied by a gradual deepening of the state of functional depression even apart from further absorption or redistribution of the ether in the body. This fact emphasizes the point that ether anesthesia is a state of coma, something quite different from normal sleep, and distinctly abnormal. The duration of the administration of an anesthetic thus plays a part in determining the resultant physiological state. This is illustrated by Experiment 6 in which an animal was maintained in ether equilibrium at a level sufficient to produce deep anesthesia. During the 9 hours of inhalation it was necessary to lower the concentration of ether inspired three times and at decreasing periods, to prevent death from respiratory failure. At the end of this time the animal was maintained in deep anesthesia by inhalation of an ether concentration ordinarily only sufficient to produce light anesthesia.

Administration of ether of this duration to human patients is rare and probably never occurs in the state of full equilibrium such as was the case in the experiment presented here.

The maintenance of ether equilibrium with a uniform concentration of inhaled ether is complicated further by variation in body temperature. A decrease in temperature commonly results during long etherizations from uncompensated heat loss. Alteration in temperature involves a change in the ratio of distribution of ether between blood and air. With decrease in temperature the tissues are capable of taking up more ether at the same concentration of inhaled ether; as a consequence a period of absorption occurs and is accompanied by a rise in the content of ether in the blood. Notwithstanding this rise, the tension exerted by the ether dissolved in the blood or tissues does not increase, since it is limited and cannot exceed the tension in the inspired air except during the process of elimination.

If the physiological response to ether is determined by the quantity of ether in the blood or tissues, then the state of anesthesia should be deepened during equilibrium maintained to a constant concentration of ether and with decrease in body temperature. On the contrary, if the physiological response is determined by the tension of ether existing in the blood or tissues, then temperature variation should be without effect. Tension seems to the writer the more probable.

In all the experimental work in which an attempt has been made to establish ether equilibrium, the body temperature was maintained nearly uniform as indicated by repeated rectal temperature readings. A hot water table and blankets were used.

In Experiments 1, 2, and 3, which are given below, dogs were brought to equilibrium with a definite concentration of inhaled ether determined by analysis of air and blood. The attainment of the desired condition was expedited by a preliminary period during which the animals were allowed to inhale a much higher concentration of the anesthetic. The existence of equilibrium was proved by the agreement in the ether content of the blood drawn from the femoral artery and that from the right heart.

The animal of Experiment 1 presents a light or moderate anesthesia with corneal reflex present when in equilibrium with a concentration of 0.079 gm. of ether per liter of air. This concentration of ether is equivalent to a tension of 20.9 mm. and is 2.94 per cent by volume. The concentration of ether in the blood was 1.14 gm. per liter.

In Experiment 2, the animal was saturated with ether at an inhaled concentration of 0.08 gm. per liter. This concentration is equivalent to a tension of 21.1 mm. and is 2.97 per cent by volume. The concentration of ether in the blood was 1.22 to 1.24 gm. per liter. The animal presented a state of moderate anesthesia with absence of corneal reflex.

In Experiment 3, the animal was in very deep anesthesia when saturated with a concentration of ether of 0.11 gm. per liter. This is a tension of 29.1 mm. or 4.09 per cent by volume. The blood concentration was 1.51 to 1.52 gm. per liter. After 3 hours of ether inhalation it was necessary to decrease the concentration inhaled to 0.08 gm. per liter of air and later to 0.07, and then 0.06 gm. per liter to prevent respiratory failure. The animal maintained a deep anesthesia throughout these changes, although the concentration in the blood fell with the decrease in concentration of inhaled ether.

Experiment 1.

Dog, female, weight 4 kilos. Animal arranged to inspire from gasometer filled with mixture of ether vapor and air. Blood samples drawn from femoral artery and from right heart. Concentration of 0.5 gm. per liter used until animal gave indication of respiratory depression. Concentration then changed and maintained at 0.079 gm. per liter (38°, 760 mm.)

Time. min.	Ether content of blood.		Concentration of ether inhaled. 38°, 760 mm. gm. per l. air	Condition of animal.
	Arterial. gm. per l. blood	Right heart gm. per l. blood		
0	0	0	0	Normal.
18	1.62	1.24	0.5	Very deeply anesthetized.
18			0.079	
60	1.14	1.16	0.079	Corneal reflex present. Light anesthesia.
120	1.13	1.12	0.079	" "
180	1.14	1.14	0.079	" "
240	1.13	1.14	0.079	" "

A concentration of 0.079 gm. of ether per liter of air is 2.94 per cent of an atmosphere.

Experiment 2.

Dog, weight 5.1 kilos. Procedure similar to Experiment 1.

Time.	Ether content of blood.		Concentration of ether inhaled. 38°, 760 mm.	Condition of animal.
	Arterial.	Right heart.		
<i>min.</i>	<i>gm. per l. blood</i>	<i>gm. per l. blood</i>	<i>gm. per l. air</i>	
26	1.56	1.30	0.5	Respiration failing.
26			0.08	
60	1.24	1.26		Corneal reflex absent. Light to moderate anesthesia.
120	1.22	1.22	0.08	" "
240	1.23	1.24	0.08	" "

Ether administration stopped.

10	0.92	0.83		Conscious, but not capable of mind.
35	0.46	0.43		Conscious.
60	0.26	0.22		

A concentration of 0.08 gm. of ether per liter of air is 2.97 per cent of an atmosphere.

Experiment 3.

Dog, male, weight 4.3 kilos. Procedure similar to Experiments 1 and 2.

Time.	Ether content of blood.		Concentration of ether inhaled. 38°, 760 mm.	Condition of animal.
	Arterial.	Right heart.		
<i>min.</i>	<i>gm. per l. blood</i>	<i>gm. per l. blood</i>	<i>gm. per l. air</i>	
0	0	0	0	Normal.
32	1.62	1.46	0.51	Respiratory failure.
60	1.50	1.47	0.11	Deep anesthesia. Corneal reflex absent.
120	1.51	1.52	0.11	" "
180	1.52	1.52	0.11	" "
240	1.50	1.52	0.11	" "
300	1.51	1.51	0.08	Respiration very depressed and animal in bad condition.
360	1.31	1.34	0.08	Very deep anesthesia and breathing somewhat depressed.
420	1.24	1.26	0.07	Breathing very shallow.
480	1.19	1.21	0.06	Condition better, very deep anesthesia.
540	1.10	1.13	0.06	Deep anesthesia.

A concentration of 0.11 gm. of ether per liter of air is 4.09 per cent of an atmosphere.

Physiological Effects of Concentrations of Ether Below the Anesthetic Tension.

The ether content of the blood drawn from the internal jugular vein offers a means of determining the degree of the saturation of the brain with ether. With the various concentrations of ether so found, the physiological responses of the subject, are readily correlated.

In Experiments 4, 5, and 6, dogs were allowed to breathe a uniform mixture of ether vapor in air. Blood samples were drawn at frequent intervals from the internal jugular vein and the ether content was determined.

Unconsciousness appears when the blood reaches an ether concentration of 0.39 to 0.46 gm. per liter. During elimination of ether, consciousness returns when a similar blood concentration is reached. The tension or partial pressure of ether corresponding to this concentration is 6.7 to 7.9 mm. These figures are in close agreement with those of White (3).

The corneal reflex disappears when the blood from the internal jugular vein contains 1.15 to 1.25 gm. of ether per liter. The corresponding tensions are 20.9 to 21.8 mm. The concentration of ether which results in abolition of the corneal reflex in these experiments is further confirmed by the findings of Experiments 1 and 2, in which complete equilibrium was reached. In the first of these, with a blood content of 1.14 gm. of ether per liter, the corneal reflex was present, while in the second with a blood content of ether of 1.22 gm. per liter, the corneal reflex was absent.

In the experiments given below, respiratory augmentation was found to commence when the blood contained 0.18 to 0.24 gm. of ether per liter. The corresponding tension is 3.1 to 4.1 mm. The respiratory volume returned to approximately the resting level when the blood contained 0.80 to 1.21 gm. of ether per liter, with corresponding tensions of 13.8 to 20.9 mm. Noticeable depression of respiration occurred when the blood reached 1.45 to 1.50 gm. per liter. The tension at this level is 25.1 to 25.9 mm. Respiratory failure occurred when the blood contained 1.54 to 1.70 gm. of ether per liter, to which the corresponding tension is 26.6 to 30.0 mm.

Because of the complexity of the mechanisms involved in the respiratory response to ether, the respiratory changes induced by

various concentrations of ether are subject to wide variation under different conditions.

Experiment 4.

Dog, male, weight 13 kilos. The internal jugular vein was exposed under cocaine anesthesia. Ether was administered by allowing the animal to breathe from a spirometer a mixture of 2 gm. of ether per liter of air. Blood was drawn from the internal jugular vein by means of a Luer syringe and analyzed for ether. The condition of the animal was noted at the same time that the blood sample was drawn.

Time.	Ether content of blood from internal jugular.	Volume of breathing.	Condition of animal.
<i>min.</i>	<i>gm. per l.</i>	<i>l. per min.</i>	
0		4.9	
6	0.24	5.4	
11	0.42	13.2	Unconscious.
27	0.81	7.1	Light anesthesia.
35	0.92	6.0	" "
45	1.06	5.0	
55	1.19	4.6	Corneal reflex absent. Muscular relaxation.
65	1.28	4.6	
75	1.36	4.5	Very deep anesthesia.
85	1.40	4.5	
95	1.47	4.2	
105	1.54	3.9	Depressed breathing.
114	1.61	0	Respiration stopped.

Experiment 5.

Dog, female, weight 7 kilos. Procedure identical with Experiment 1.

Time.	Ether content of internal jugular blood.	Volume air breathed.	Condition of animal.
<i>min.</i>	<i>gm. per l.</i>	<i>l. per min.</i>	
0	0	3.1	Normal.
3	0.18	5.2	
6	0.39	10.7	Unconscious.
15	0.70	8.1	Light anesthesia.
25	0.93	4.0	
35	1.03	3.6	
40	1.21	3.0	Corneal reflex absent.
50	1.35	2.9	Muscular relaxation.
60	1.46	2.9	
70	1.54	1.6	
80	1.62	1.0	Respiration depressed.
87	1.70	0	Respiratory failure.

Experiment 6.

Dog, male, weight 6.5 kilos. Procedure same as in previous two experiments.

Time.	Ether content of internal jugular blood.	Volume of air breathed.	Condition of animal.
<i>min.</i>	<i>gm. per l.</i>	<i>l. per min.</i>	
0	0	3.4	Normal.
5	0.3	6.7	
10	0.46	13.4	Unconscious.
20	0.74	8.2	
30	0.95	6.1	
45	1.17	3.3	Corneal reflex absent.
60	1.38	3.3	
75	1.45	2.4	
90	1.54	0	Respiration failure.

SUMMARY.

1. When the body is in complete equilibrium with any concentration of inhaled ether, the ether content of the blood drawn from any source serves as an index of the condition.

2. The state of equilibrium is established when the mass of ether in the inspired air is identical with that in the expired air or when the ether contents of blood from the right heart and of arterial blood are the same.

3. In any condition short of complete equilibrium, venous blood drawn from any source other than the right heart has no precise significance as to the degree of saturation of the body as a whole. The ether content of venous blood from any organ or structure indicates the state of saturation of that structure only.

4. The ether content of blood from the internal jugular vein is an index of the degree of saturation or concentration in the central nervous system, upon which depends the degree of anesthesia.

5. During elimination of ether the ether content of the arterial and venous blood drawn from any source differs by only a very slight margin and for this reason the physiological response to ether may be readily determined with fair precision during elimination; as it cannot be during induction.

6. The physiological response to various concentrations of ether as presented in this paper is in close agreement with those of other recent workers in this field and indicate that the anesthetic tension is 3.7 to 4.0 per cent of an atmosphere. The commonly accepted figure (7 per cent), as determined by Boothby, is probably nearly twice as great as it should be. It is commonly found that mixtures of air and ether vapor, made up to contain a desired amount of ether per liter, actually on analysis, prove to contain much less. The fact that the amount found chemically is often very close to one-half of that expected, may or may not be of some significance.

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THE ABSORPTION, DISTRIBUTION, AND ELIMINATION OF ETHYL ETHER.

V. THE IMPORTANCE OF THE VOLUME OF BREATHING DURING THE INDUCTION AND TERMINATION OF ETHER ANESTHESIA.

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Rapid induction of ether anesthesia is desirable for the reason that the prolongation of the subanesthetic stages is detrimental to the subject. In order to induce rapid anesthesia it is the practice to administer a concentration of ether vapor much higher than that necessary to maintain fully developed anesthesia. This procedure requires skill upon the part of the anesthetist; it also results in a great increase in the irritant action upon the lungs.

The following discussion deals with the action of ether as a pulmonary irritant and presents a method whereby induction can be made rapid without the employment of high concentrations of ether vapor.

Action of Ether as a Pulmonary Irritant.

Ether vapor is unquestionably an irritant. Its action elicits from the respiratory tract the response common to all respired irritants; it causes reflex coughing by laryngeal irritation and under suitable conditions inhibition of respiration; it produces increased mucous flow through the stimulation of the glands of the respiratory mucosa. Its irritant action upon the cornea and conjunctiva is known so well as to require no comment.

As an irritant ether presents certain peculiarities which differentiate it from the common lung irritants, nevertheless, a brief summary of the general characteristics of pulmonary irritants

tends to throw light upon this phase of the action of ether. A complete summary of the action of pulmonary irritants in general has been recently published by the writer (1).

The intensity of the action of any irritant is determined by the particular mode of its action upon the tissues with which it is brought in contact, the delicacy of the tissue upon which it exerts its action, the concentration in which the irritant is present, and to some extent upon the duration of its action.

The seat of action of the ether, like that of other irritants which are not decomposed in their passage through the respiratory tract, is determined by its solubility. The more soluble the irritant the greater the damage to the upper respiratory tract since it is there that a highly soluble irritant is largely removed from the air through contact with the moisture upon the first surfaces with which it comes in contact. For this reason the irritation caused by the inhalation of such soluble substances as ammonia or formaldehyde is confined almost entirely to the upper respiratory passages. Less soluble irritants pass more deeply into the respiratory tract since it requires a more extensive and prolonged exposure to the moist walls to absorb them. In this respect ether is peculiar in that not alone does it have a comparatively low solubility in water for an irritant (ether 1:15; ammonia 1:444; hydrochloric acid 1:385, etc.), but in consequence of the fact that ether is not destroyed by the tissues, the continued inhalation brings fluids, with which it comes in contact, to saturation with the tension of ether to which they are exposed. Since absorption of ether through the bronchi and bronchioles must be comparatively sluggish, these structures are bathed continually during inhalation with a concentration of ether only slightly below that of the inhaled concentration. In contrast to the high concentration to which the bronchi and bronchioles may be exposed, the more delicate alveolar structures are relatively spared since the active absorption which occurs there prevents the tension from rising appreciably higher than that in the arterial blood. This in turn is closely limited by the resultant physiological activities produced by the absorbed and circulating ether. No matter how great a concentration of ether acts upon the respiratory passages, that effective in the alveoli cannot rise above a very definite level since the high arterial content causes stoppage of breathing. Thus the most

vulnerable portion of the lung is to a large extent spared from the irritant action of the inhaled ether, while the bronchi and bronchioles bear the brunt of the action.

The activity of an irritant does not increase merely in direct proportion to concentration, but at a greater rate. Thus, a comparatively slight increase in the concentration of ether inhaled may result in a considerable increase in the resultant irritation.

The pharmacological character of ether makes it difficult to study directly its irritant action through the respiratory reflexes elicited. The coughing, laryngeal closure, and respiratory inhibition immediately following the inhalation of a high concentration of ether vapor rapidly disappear with the continuation of the inhalation, since the threshold of the reflexes is raised by the action of the absorbed anesthetic. For this very reason it is customary to initiate the inhalation of ether gradually and to force the concentration as the threshold of the reflexes is raised and their action thus avoided. To a similar end the preadministration of morphia or the use of nitrous oxide during induction allow the forcing of the concentration without interference by the respiratory reflexes, coughing, etc. The presence or absence of these protective reflexes has nothing whatever to do, however, with the actual bronchial or bronchiolar irritation resulting from the ether, and the occurrence of a subsequent pneumonia.

The increased activity of the mucous glands of the respiratory tract, resulting from the action of irritants, affords in many instances a mode of protection. Such substances as chlorine or hydrochloric acid gas are partially neutralized by the alkaline mucous secretions. As a result the underlying tissues are spared to some extent. With ether this is not the case. Ether is neither neutralized nor destroyed by the mucous coating which forms on the respiratory structures. The small amount of ether absorbed by it is rapidly replaced from the inspired air during the continuation of the inhalation. The tissues covered by the mucous coating are thus bathed with a fluid saturated with ether at the tension inhaled.

Rapid Ether Induction by Means of Increased Respiratory Volume.

The best and indeed the only way to limit the irritant action of ether vapor upon the respiratory tract is through the employment

of an ether tension little, if any, higher than the anesthetic tension. To induce anesthesia under such a condition is, however, a very prolonged and difficult procedure since the rate of absorption is very slow.

The employment of concentrations of ether higher than the anesthetic during the induction period is explained by the fact that the rate at which ether is absorbed and, what is equally important for induction, the level of ether in the arterial blood, are proportional to the concentration of ether inhaled. The time of induction of anesthesia is thus more than proportionately shortened.

An equally rapid induction can be brought about without elevating the concentration of the inhaled anesthesia by increasing the volume of air breathed. The rate of ether absorption and the level reached in the arterial blood is nearly proportional to the volume of pulmonary ventilation.

It is entirely feasible by means of inhalation of dilute carbon dioxide to produce a fivefold increase in the volume of breathing. With respiration increased to this extent and under the inhalation of ether vapor at the anesthetic tension, induction is brought about in nearly the same time as would result under the inhalation of an ether concentration five times as great as the anesthetic tension and with the normal volume of breathing. Doubtless a part of the advantage of induction by nitrous oxide is due to the increased breathing.

A fivefold augmentation of respiration by means of the inhalation of 5 to 10 per cent carbon dioxide in air or oxygen is an entirely safe procedure. The safety of this inhalation is assured by the present wide use of such a dilute mixture of carbon dioxide for the resuscitation after carbon monoxide poisoning (2, 3), and also as a means of rapid deetherization after operation (4, 5).

Experiment 1 illustrates a typical ether induction on a dog by means of the inhalation of a moderate concentration of ether. Experiment 2 shows induction produced by means of a low concentration of ether (one-half that of the previous experiment), but with the respiratory volume augmented by means of inhalation of dilute carbon dioxide. The time required to produce anesthesia in these two experiments was approximately the same, although the concentrations inhaled were widely different. The dog of Experiment 1, to which the higher concentration of ether

vapor was administered, presents an irregular hyperpnea soon after the commencement of inhalation, which resulted in a period of very rapid ether absorption and rendered it necessary to discontinue the inhalation for a time to prevent respiratory failure. The occurrence of hyperpnea during the inhalation of ether concentrations above the anesthetic level renders such inhalations a dangerous procedure in the hands of one who is not skilled. The dog of Experiment 2 received carbon dioxide and the respiratory volume at once rose to a level four to five times as great as the resting. The elevated respiratory volume was maintained with no irregularity and with slight progressive decrease as anesthesia developed.

The influence which the increase in the respiratory volume exerts upon the rate of absorption and level of ether in the arterial blood is greatest at the beginning of the inhalation and decreases as the absorption progresses. As a result of this it is not necessary to continue the administration of the dilute carbon dioxide after full anesthesia has developed.

Experiment 3 illustrates induction produced by means of the combination of augmented respiratory volume induced by CO_2 and a concentration of inhaled ether above the anesthetic tension. The combination of the two factors instrumental in increasing the rate of absorption results in a very rapid induction and, furthermore, avoids the danger of irregular absorption due to uncontrolled respiration. Although the high concentration of ether inhaled must cause more pulmonary irritation than is the case when the anesthetic tension is employed, this effect is at least partially compensated for by the relatively brief time over which the high concentration is inhaled.

The apparatus used in the experiments cited here to administer ether together with carbon dioxide, was designed for quantitative experimental purposes and is not intended, nor practicable, for the general administration of ether anesthesia. There are available, however, two types of inhalators for the administration of dilute carbon dioxide in air (2) or in oxygen (3) and these could be adapted quite feasibly for the administering of ether anesthesia. It is hoped that opportunity may be found for the practical development of such an apparatus.

Showing Ether Induction under Moderate Concentration.

Dog, female, weight 8 kilos. No preliminary morphinization. Breathing ether concentration, 0.3 gm. per liter. General procedure similar to previous experiments.

Time.	Volume of air breathed. 20°, 760 mm. wet.	Ether content of blood.		Ether absorbed and eliminated.	Remarks.
		Arterial.	Right heart.		
min.	l.	gm. per l.	gm. per l.	gm.	
0	3.1	0	0	0.0	Ether started.
10	15.5	1.21	0.48	4.0	Light anesthesia.
20	5	1.6	1.11	10.6	
40	2.9	1.64		12.1	
50	1.8	1.71	1.47	12.8	Very deep anesthesia. Respiration stopped.
52					

Administration stopped and elimination started.

10	11.9	0.82	1.06	3.0	Complete consciousness.
20	9.2	0.42	0.58	7.4	
30	2.8	0.20		8.2	
40	3.6	0.14		9.5	
50	1.8	0.08		10.2	
60	2.2			10.6	
70	2.2			10.9	
80	2.1			11.1	
80					

Experiment 2.

Showing Rapid Induction by Low Concentration of Inhaled Ether, When Respiration Is Augmented by CO₂ Inhalation.

Dog, male, weight 7.5 kilos. No preliminary morphinization. Breathing ether concentration of 0.1 gm. per liter with inhalation of 7.5 per cent carbon dioxide throughout.

Time.	Volume of air breathed. 20°, 760 mm. wet.	Ether content of arterial blood.	Ether absorbed.	Remarks.
min.	l.	gm. per l.	gm.	
0	2.8	0	0	CO ₂ inhalation started.
5	10.6	0	0	Inhalation of 0.1 gm. per liter of ether.
15	10.6	0.81	4.0	Unconscious.
35	10.2	1.32	7.9	Very deep anesthesia.
55	8.4	1.39	9.8	
75	6.2	1.41	10.6	
95	6.1	1.43	11	
115	5.8	1.43	11	

Elimination started.

0				
10	9.6	0.62	5.3	Conscious.
30	11.4	0.16	9.7	CO ₂ inhalation stopped.
60	3.4	0.06	10.1	

Experiment 3.

Showing Prolonged Anesthesia under Low Concentration of Ether with Induction and Recovery under Augmented Breathing Induced by CO₂.

Dog, male, weight 9 kilos. Same animal as used for Experiment 4. No preliminary morphinization. Breathing ether concentration of 0.2 gm. per liter until anesthesia was induced and after that 0.1 gm. per liter. General procedure similar to previous experiments.

Time.	Volume breathing. 20°, 760 mm. wet.	Ether content of arterial blood.	Ether absorbed and elimi- nated.	Remarks.
min.	l.	gm. per l.	gm.	
0	3.0	0	0	CO ₂ inhalation, 7.5 per cent started.
5	14	0	0	Inhalation 0.2 gm. per liter.
0		0	0	Ether started.
3				Anesthesia.
10	13	1.48	7.1	Changed inhalation of ether to 0.1 gm.
20	12.4	1.25	8.6	
30	12.2	1.41	10.1	CO ₂ inhalation stopped.
40	4.2	1.43	11	
60	2.3	1.46	12.4	
80	2.8	1.47	13	
100	2.6	1.46	12.7	

Elimination started.

0				CO ₂ inhalation reestablished.
10	11	0.51	5.2	Conscious.
20	14.2	0.2	9.6	CO ₂ inhalation discontinued.
30	3.4	0.16	10.4	
40	3.1	0.09	11.1	
50	3.3		11.4	

CONCLUSIONS.

1. Prolonged induction of ether anesthesia is undesirable. By increasing the concentration of ether the induction of anesthesia can be greatly accelerated, but the inhalation of high concentrations of ether results in an even more than proportionately great pulmonary irritation.

2. Rapid induction of anesthesia can be brought about and pulmonary irritation reduced to a minimum by the use of low concentrations of ether, but with increased volume of breathing induced by dilute carbon dioxide. The elimination can be similarly accelerated, as White (5) has already demonstrated on man.

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STUDIES ON PROTEINOGENOUS AMINES.

XVI. THE EXCRETION OF IMIDAZOLES IN THE URINE UNDER NORMAL AND PATHOLOGICAL CONDITIONS WITH SPECIAL CONSIDERATION OF NEPHRITIS.*

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INTRODUCTION

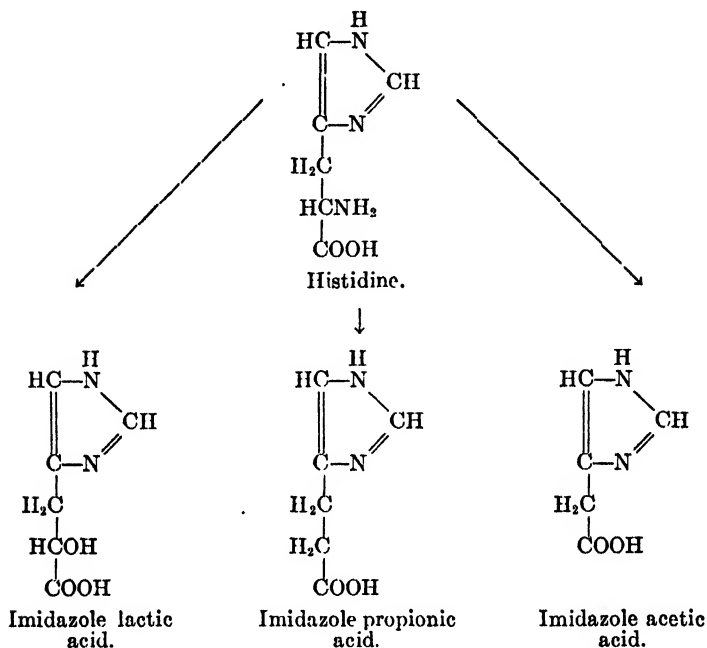
The study of the intermediary metabolism of the amino acids has, for some time, been a problem of great interest to the biochemist and the clinical physiologist. Of the cyclic complexes of the protein molecule, the phenyl and oxyphenyl derivatives of alanine have been most frequently studied experimentally, probably on account of the great resistance which the benzene ring offers to disruption in the body and the comparative ease with which benzene derivatives can be isolated and identified.

A second group of cyclic compounds, the imidazoles, has received scantier attention, probably because the imidazole complex is rather easily disrupted by living protoplasm and the products formed cannot be easily identified. This seems most unfortunate when we consider the preeminent position of histidine as a constituent of protoplasm. Thus, besides its almost constant presence in animal and vegetable proteins, histidine is present to the extent of about 10 per cent in hemoglobin and about 30 per cent of the total extractive nitrogen of skeletal muscle is due to carnosine (β -alanyl-histidine).

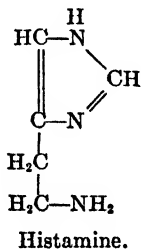
The catabolism of histidine by unicellular organisms, *e.g.* bacteria, may lead to any or all of the following types of reaction.

* For the articles of this series previously published see the *J. Am. Chem. Soc.*, 1918, xl, 1716; *J. Biol. Chem.*, 1919, xxxix, 497, 521, 539, 585; 1920, xliii, 521, 527, 543, 557, 567, 579; 1922, 1, 131, 193, 235, 271.

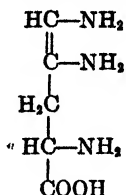
1. *Deamination*.—A process that may lead to the formation of imidazole lactic, propionic, or acetic acids.



2. *Decarboxylation*.—A process that leads to the formation of histamine, β -iminazolyethylamine, a substance that has recently aroused considerable interest on account of its powerful physiological activity.



3. *Disruption of the Imidazole Ring.*—This may lead to a final complete disruption of the molecule with liberation of ammonia, or may produce a series of hitherto unstudied amines of the type



Body cells may also be capable of either or all of these types of enzymatic activity. It is certain that by far the largest part of the imidazole compounds do not find their way into the urine as such. The imidazoles excreted in the urine represent only a small fraction of the histidine ingested. This may be explained in one of three ways.

1. The imide hydrogen, position 1, of the imidazole ring, may have been replaced by some less mobile radical (such as CH_3). Such a derivative would give no color with an alkaline solution of *p*-phenyldiazonium sulfonate. Since this color reaction is usually employed for the detection and estimation of imidazoles, the above substituted derivatives would not be estimated although they are true imidazoles.

2. The imidazole ring is disrupted by tissue protoplasm as it is disrupted by bacterial activity.

3. Imidazoles may be excreted in the feces.

It is impossible to say which of these explanations is correct until the subject has been further investigated.

Several attempts have been made to isolate and identify the imidazole complexes that are found in urine. Thus Engeland¹ succeeded in isolating a small amount of histidine, $\text{C}_2\text{N}_2\text{H}_3\text{-CH}_2\text{-CHNH}_2\text{-COOH}$, and a small amount of imidazole aminoacetic acid, $\text{C}_2\text{N}_2\text{H}_3\text{-CHNH}_2\text{-COOH}$, from 40 liters of urine. By far the largest part of the imidazole complexes could not be identified. Urocanic acid, $\text{C}_2\text{N}_2\text{H}_3\text{-CH=CH-COOH}$, has been found, occasionally, in the urine of dogs.² Fürth³ has recently conducted

¹ Engeland, R., *Z. physiol. Chem.*, 1908, lviii, 49.

² Jaffé, M., *Ber. chem. Ges.*, 1874, vii, 1609; 1875, viii, 811.

³ Fürth, O., *Biochem. Z.*, 1919, xcvi, 269.

a series of isolation experiments with human urine that did not lead to the identification of a single imidazole compound. It seems, then, that the imidazole fraction of urine contains a variety of histidine catabolism products. These may, in fact, vary for different individuals.

Although we do not know with what the imidazole complex is combined as it appears in the urine, *we can estimate the total quantity of the imidazole complexes that are excreted.* Imidazole compounds have long been known to give an orange-red color when they are mixed with an alkaline solution of *p*-phenyldiazonium sulfonate. In 1914 Weiss and Ssoblew⁴ published a method for the estimation of histidine which was based upon the well known reaction of Pauly.⁵ This method was subsequently applied by Masslow⁶ in a series of metabolism experiments on dogs and by Fürth³ to a somewhat similar series on man. The urine was treated with an excess of baryta. The filtrate was freed from barium with H_2SO_4 . The filtrate from the BaSO_4 was then neutralized with Na_2CO_3 , the resulting solution concentrated somewhat and the liquid then subjected to a series of comparative Pauly reactions from which the concentration of imidazoles was estimated. Since the work of these investigators is to be considered in detail somewhat later, we wish merely to call attention to the fact that they were using a crude, incompletely fractionated urine for their colorimetric determinations. Their test solutions must have contained urochromogen, urochrome, polyphenols, and the aromatic hydroxy acids, all of which would contribute to the color obtained with the alkaline diazo reagent.

This brings us to a consideration of the "diazo reaction" of urine, a reaction that has been shrouded in uncertainty since the time of its introduction by Ehrlich⁷ in 1882. When any sample of urine is treated first with a solution of *p*-phenyldiazonium sulfonate and then with ammonia, an orange-red color of varying intensity is produced. In certain pathological conditions, *e.g.* typhoid fever, a brilliant Bordeaux red color is produced which is

⁴Weiss, M., and Ssoblew, N., *Biochem. Z.*, 1913-14, lviii, 119.

⁵Pauly, H., *Z. physiol. Chem.*, 1904, xlii, 508; 1905, xlv, 159.

⁶Masslow, M., *Biochem. Z.*, 1915, lxx, 306.

⁷Ehrlich, P., *Z. klin. Med.*, 1882, v, 285; *Char. Ann.*, 1883, viii, 140; *Deutsch. med. Woch.*, 1883, ix, 549; 1884, x, 419.

also present in the foam when the mixture is shaken. The latter reaction was considered positive by Ehrlich; but since all samples of urine give *some* color, it is only by experience that one is able to distinguish an abnormal pathological reaction from one that is normal. Urine may give two distinct kinds of diazo reaction: one that is invariably obtained with normal and pathological urines and which appears most brilliantly when sodium carbonate is used instead of ammonia; and the other, the true Ehrlich diazo reaction, that is obtained only with pathological urines and is characterized by a red foam coloration. This confusing state of affairs has recently been clarified by the work of Weiss.⁸

Fractionation of the Urine.

Weiss divided the urine into three fractions with the aid of lead acetate as follows:

Fraction I. The Urobilin Fraction.—When urine is treated with an excess of neutral lead acetate a precipitate is obtained that contains the lead salts of uric, carbonic, phosphoric, and sulfuric acids, colorless urobilinogen, red urobilin, indole chromogens, uroerythrin, and, in pathological cases, may contain alkapton chromogens. Three-fourths of the urinary coloring matter appears in this fraction. Of this, about 10 per cent is due to urochrome, which, although it properly belongs in fraction II, is adsorbed by the voluminous precipitate. When fraction I is purified, so that it is free from adhering fraction II, it does not give the positive diazo test of Ehrlich. The chromogens of this fraction are sharply differentiated from those of fraction II by the fact that the lead salts are insoluble in dilute acetic acid and the free chromogens are precipitated from water solution by ammonium sulfate.

Fraction II. The Urochrome Fraction.—When sodium hydroxide is added to the filtrate from fraction I, a precipitate is obtained that consists largely of lead hydroxide, but that also contains some of the purines, the aromatic hydroxy acids, urochromogen, and the yellow urochrome. When sufficient lead acetate and exactly the right amount of sodium hydroxide have been used, the precipitate contains all the remaining urinary coloring matter, the filtrate

⁸ Weiss, M., *Biochem. Z.*, 1920, cii, 228; cxvii, 61.

from fraction II being colorless. The two most interesting constituents of this fraction are urochrome and its precursor urochromogen.

Urochrome is yellow. It gives an orange-red color when it is mixed with an alkaline solution of *p*-phenyldiazonium sulfonate, but the foam obtained is colorless. This compound, therefore, gives a positive Pauly reaction, but a negative Ehrlich reaction. All normal urines contain urochrome.

Urochromogen is pale yellowish green in color. It can be readily oxidized to the yellow urochrome, hence it reduces permanganate and ammoniacal silver nitrate. *Urochromogen gives a Bordeaux red color with an ammoniacal solution of p-phenyldiazonium sulfonate and a red foam. This is the compound that is responsible for the diazo reaction of Ehrlich* (according to Weiss). It is almost absent from normal urine; but may occur in quantity under certain pathological conditions. Weiss concluded that urochromogen is polyphenolic in character and that these polyphenols are the diazo chromogens of Ehrlich. Hermanns and Sachs⁹ have recently studied the diazo chromogens of pathological urines in tuberculosis and cancer and conclude that the composition of the diazo chromogens of Ehrlich is not constant, they are phenolic in character, and they appear to be derivatives of tyrosine or tryptophane. Although Hermanns and Sachs do not believe, with Weiss, that urochromogen is the diazo chromogen of Ehrlich, their findings are in essential agreement with those of Weiss as to the phenolic character of the diazo chromogens. We wish especially to stress the fact that our experiments were carried out *not* on this pathological phenolic fraction but on the *phenol-free, true imidazole fraction*. Our mode of procedure removes the phenols, both normal and pathological.

Fraction III. The Imidazole Fraction.—The filtrate from the insoluble lead salts of fraction II is fraction III. Among other things, this fraction contains the imidazole derivatives. It gives a brilliant Pauly diazo reaction, but does not give the positive Ehrlich diazo reaction. This is, obviously, the fraction that should be studied if we wish to shed light on the metabolism of

⁹Hermanns, L., and Sachs, P., *Z. physiol. Chem.*, 1921, cxiv, 79, 88. Hermanns, L., *Z. physiol. Chem.*, 1922, cxxii, 98.

histidine in the mammalian organism. The method originally employed by Weiss and Ssobolew⁴ and subsequently adopted by Masslow⁶ and Fürth⁸ is bound to give erroneous results because these investigators determined the *total* diazo value of the urine, which includes the aromatic oxy acids, most of the purines, urochromogen, and urochrome. The total, unfractionated whole urine also contains a group of substances that interfere with the color production. To overcome this interference, the above investigators found it necessary to use varying and frequently large portions of diazo reagent and sodium carbonate. Under these conditions an appreciable autogenous color always develops that will, of course, raise the results.

In 1919 we published a method for the quantitative colorimetric estimation of imidazoles.¹⁰ At that time we stated that the method could not be applied to whole urine because certain interfering substances prevented the proper development of the color. After the appearance of Weiss' paper we resumed our work on urine, applying the method to his fraction III, the true imidazole fraction. We were gratified to find that the lead treatment had not only removed all of the extraneous, non-imidazole diazo chromogens, but the interfering substances as well. We are able, therefore, to present a simple, rapid, and accurate method for the estimation of imidazoles in urine.

MODE OF PROCEDURE.

Of the exactly measured 24 hour specimen of urine, 100 cc. are transferred to a 250 cc. Pyrex flask and treated with 25 cc. of a saturated solution of lead acetate. A precipitate is obtained that contains the lead salts of uric, carbonic, phosphoric, and sulfuric acids, colorless urobilinogen, red urobilin, indole chromogens, uroerythrin, and, in pathological cases, may contain alkapton chromogens. The insoluble lead salts are *not* filtered off. The mixture is treated with a 10 per cent solution of sodium hydroxide until the supernatant liquid is colorless and until test portions of this liquid give only the faintest cloud with sodium hydroxide. From 12 to 18 cc. of the sodium hydroxide solution are usually required. An excess of alkali must be carefully avoided for this

¹⁰Koessler, K. K., and Hanke, M. T., *J. Biol. Chem.*, 1919, xxxix, 497.

redissolves the chromogens. A urine sample is occasionally obtained that contains so much coloring matter that 25 cc. of the lead solution is insufficient to effect a complete precipitation. In such cases it is necessary to add a second and at times even a third 25 cc. portion of the lead acetate solution to remove the coloring matter and the interfering substances completely. This secondary precipitate contains some of the purines, the aromatic hydroxy acids, urochromogen, and urochrome. The mixture is allowed to settle for a few hours. The supernatant liquid should be clear and *colorless*. The mixture is filtered with suction, using a Buchner funnel, the precipitate well washed with water, and the filtrate treated with an excess of disodium hydrogen phosphate which removes the lead completely. The slightly alkaline filtrate from the lead phosphate is transferred to a glass dish and concentrated on the water bath to a volume of 10 to 20 cc. *This removes the volatile phenols completely.* The residue is diluted with water to exactly 200 cc. Of this colorless to pale brown liquid, 0.10 to 1.0 cc. portions are taken for the colorimetric estimation of imidazoles which is carried out exactly as previously described by us.¹⁰

The Congo red-methyl orange (CR-MO) standard¹⁰ is used for comparisons. The color obtained matches that of the standard perfectly in most cases. From 3 to 5 minutes are required to obtain a color of maximum intensity, the color being permanent for about 5 minutes during which time the readings can be easily made. The color obtained is directly proportional to the amount of test liquid employed. This is proof positive that all the interfering substances have been removed by the lead treatment.

Calculation of Results.

The most carefully conducted isolation experiments have failed to reveal the identity of the major part of the imidazole complexes. To convert colorimetric readings, by means of a table, to any of the known imidazoles would, obviously, be incorrect. *We have decided to calculate our results on the basis of a hypothetical compound and to refer to the values obtained as the imidazole value.* The urinary imidazole complexes seem to be endowed with acid as well as basic properties, which is apparent from the following facts. They form alcohol-insoluble barium salts and they cannot

be extracted with amyl alcohol from a solution made alkaline with sodium hydroxide. Because of this fact we have decided to use the mean color value of the four carboxylated imidazoles, for which we have already constructed a table,¹⁰ as the basis of our calculations.

We have found that 5.0 mm. (CR-MO) of standard is equivalent to 0.00001 gm. of histidine dichloride, 11.2 mm. (CR-MO) of standard is equivalent to 0.00001 gm. of imidazole acetic acid, 13.6 mm. (CR-MO) of standard is equivalent to 0.00001 gm. of imidazole propionic acid, and 9.3 mm. (CR-MO) of standard is equivalent to 0.00001 gm. of imidazole lactic acid. 10.0 mm. (CR-MO) of standard is approximately the mean color value for 0.00001 gm. of these four carboxylated imidazoles. Each millimeter of our (CR-MO) standard comparison color is, therefore, equivalent to 0.000001 gm. of imidazole complex when the test cylinder is set at 20 mm. The concentration of the imidazole complex is then calculated from the simple linear relationship that each millimeter depth of the indicator solution corresponds to 0.001 mg. of imidazole complex.

Suppose a sample of urine, whose total 24 hour volume is 1,500 cc., has been subjected to the procedure just described and that 0.10 cc. of the final test liquid had a color value equivalent to 5.0 mm. (CR-MO). This would be equal to 0.000005 gm. of imidazole complex for the 0.10 cc. portion, 0.010 gm. for the 200 cc. of test liquid (representing 100 cc. of urine), and 0.150 gm. for the entire 24 hour sample of urine. *This urine we would speak of as having an imidazole value of 150 and such a person would be said to have excreted 150 mg. of imidazoles.*

If, at some subsequent date, the exact character of the imidazole complex is determined, all the previously acquired data can be readily recalculated in terms of the new derivative.

The Color Value of the Imidazole Fraction Is Largely Due To Imidazoles.

That the diazo chromogens of *normal* urine are predominantly imidazole in character was clearly shown by the earlier work on the oxyproteic acids and recently corroborated by the experiments of Fürth.⁸ The diazo chromogens belonging to our imidazole

fraction are not precipitated by basic lead acetate, but are precipitated by phosphotungstic acid and by silver nitrate and barium hydroxide. The exact amount precipitated by each of these reagents has not, heretofore, been determined. We found that 65 per cent of the diazo chromogens are precipitated by phosphotungstic acid from a solution containing 9 cc. of 37 per cent HCl per 100 cc. of precipitation liquid. Imidazoles are, as a rule, not quantitatively precipitated by this reagent.

One of the characteristic properties of the imino compounds is their ability to form insoluble silver complexes. This reaction is so much more characteristic for imidazoles than the phosphotungstic acid precipitation that we shall report our experiment in detail.

Imidazole compounds can be approximately separated from the purines by precipitation with silver nitrate. The purines are precipitated in acid solution while the imidazoles come down only on addition of alkali.

Normal urine—100 cc.—was precipitated as usual with lead acetate and sodium hydroxide. The filtrate was then treated with a slight excess of Na_2HPO_4 , filtered, the filtrate concentrated and finally diluted to 200 cc. 0.10 cc. of this solution had a color value equivalent to 11.7 mm. (CR-MO). The remaining test liquid was transferred with 50 cc. of water to a 500 cc. flask and treated with NO_2 -free nitric acid until distinctly acid in reaction. Silver nitrate—30 cc. of a 20 per cent solution—was then added, which produced a copious precipitate. This was filtered off and washed with water. This precipitate is referred to below as purine precipitate I. The filtrate from the purine precipitate was then treated with a further 25 cc. portion of silver nitrate solution. Barium hydroxide—50 gm. dissolved in 100 cc. of hot water—was then added. The dark brown precipitate so obtained was filtered off and washed with 100 cc. of a cold saturated solution of baryta. The filtrate was discarded. The precipitate is referred to below as imidazole precipitate II.

Each of the two silver precipitates was suspended in water and treated with an excess of 37 per cent HCl. Precipitate II was also treated with sulfuric acid to remove the barium. After filtration the filtrates were carefully neutralized with sodium hydroxide, concentrated, and finally diluted to 200 cc. The colorimetric determinations were then carried out in the usual manner.

Purine Precipitate I.

Total volume 200 cc.

0.50 cc. had a color value equivalent to 7.0 mm. (CR-MO). Match perfect.

1.00 cc. had a color value equivalent to 14.0 mm. (CR-MO). Match perfect.

This is equal to 1.4 mm. for a 0.10 cc. portion. Since the original color value was 11.7 mm. for a 0.10 cc. portion, 12 per cent of the total color value obtained originally was due to purines.

Imidazole Precipitate II.

Total volume 200 cc.

0.10 cc. had a color value equivalent to 8.7 mm. (CR-MO). Match perfect.

0.20 cc. had a color value equivalent to 17.4 mm (CR-MO). Match perfect.

This corresponds to 74.4 per cent of the total original color value.

Of the total color value, 86.4 per cent can be precipitated as silver salt; the rest must appear in the filtrate either as a soluble fraction of the same compounds or possibly as some other, non-imidazole derivative. The purines that give the diazo test are true imidazole derivatives. Burian¹¹ has shown in 1904 that the purine derivatives, in which the imide hydrogen (position 7) is not substituted and which contain the true amidine linking, give red compounds with alkaline solution of phenyldiazonium derivatives. This reaction is obtained, therefore, with xanthine, hypoxanthine, guanine, and adenine, but is not obtained with theobromine (2, 6-dioxy-, 3, 7-dimethylpurine) or with caffeine (2, 6-dioxy-, 1, 3, 7-trimethylpurine). Substitution in the pyrimidine ring does not prevent coupling, for the color reaction is given by theophylline (2, 6-dioxy-, 1, 3-dimethylpurine).

As far as the purines are concerned we are perfectly justified in including them in our imidazole fraction as imidazole derivatives although we can hardly say, with certainty, that they are derived from histidine. We cannot, however, be certain that all the diazo chromogens precipitated by silver nitrate from an acid solution

¹¹Burian, R., *Ber. chem. Ges.*, 1904, xxxvii, 696; *Z. physiol. Chem.*, 1904, xlii, 297.

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are purines for it is conceivable that certain complex histidine derivatives might give silver salts that are insoluble in a slightly acid solution.

Process Used for the Estimation of Imidazoles in Urine Is Quantitative.

200 cc. of urine were divided into two equal parts. To the one-half was added 0.010 gm. of imidazole acetic acid hydrochloride. Both fractions were then identically precipitated with lead acetate and sodium hydroxide, filtered, the filtrate freed from lead with Na_2HPO_4 , and the residue obtained on evaporation finally diluted to 200 cc. 0.10 cc. of the untreated fraction had a color value equivalent to 10.6 mm. (CR-MO), 0.10 cc. of the fraction to which the imidazole acetic acid had been added had a color value equivalent to 16.2 mm. (CR-MO).

The difference between these two readings—5.6 mm.—represents the color value due to the added imidazole acetic acid. The color obtained with imidazole acetic acid matches the (CR) standard better than it matches the (CR-MO) standard.¹⁰ The table,¹⁰ previously published, for converting colorimetric readings into grams of imidazole acetic acid was based upon the (CR) standard which has an intensity 10/11 as great as the (CR-MO) standard.¹⁰ The above figure—5.6 mm.—has, therefore, to be multiplied by 11÷10 before comparison with the table. This gives 6.16 mm. (CR) as the color value due to the imidazole acetic acid, which, by table, is equal to 0.0100 gm. for the entire 200 cc. of test liquid which equals 100 per cent of the amount originally introduced.

This experiment proves: that imidazoles are not occluded by the voluminous lead precipitate; evaporation in slightly alkaline solution does not alter the chromogenic properties of the imidazoles; the treatment with lead acetate and sodium hydroxide completely removes the interfering substances that are contained in unfractionated urine.

Relation between the Protein Content of the Diet and the Imidazole Excretion in Normal Adults.

The quantity of imidazole complex excreted in the urine during a 24 hour period was first determined for several normal adults.

The diet had a caloric value of 2,500 to 2,800, but the protein content varied from 20 to 120 gm. The fluid intake was not restricted, but it is to be noted that this varied considerably for the different individuals. The subjects were kept on each of the diets for 5 consecutive days, the 24 hour specimen being collected on the last of these days.

On a diet low in protein—20 to 50 gm.—from 118 to 140 mg. of imidazole complex were excreted in 24 hours. On a regular mixed diet, containing from 80 to 90 gm. of protein per day, 134 to 205 mg. of imidazole complex were excreted in 24 hours. When the protein content of the diet was raised to 120 gm., 172 to 219 mg. of imidazole complex were excreted. (See Table I.)

TABLE I.
Effect of Diet on Imidazole Excretion in Normal Individuals.

Name.	Diet.		Urine.	
	Character.	Protein content.	Total volume for 24 hrs.	Imidazole value.
		gm.	cc.	
M. T. H.	Low protein.	20	800	118
	Regular mixed.	90	680	134
	High protein.	120	700	172
H. L. H.	Regular mixed.	80	1,340	181
	High protein.	120	1,480	201
K. K. K.	Low protein.	30	1,520	140
	Regular mixed.	90	1,670	205
	High protein.	120	1,035	219
M. M. A.	Low protein.	50	1,210	119

The following facts, shown in Table I, are to be especially noted.

1. The imidazole complex excreted represents only a small fraction of the histidine ingested.

2. On a diet containing very little protein and, therefore, very little histidine, about 120 mg. of imidazole complex were excreted. This must be largely endogenous in origin and represents an end-product of the normal protein catabolism of body tissue.

3. On a diet containing an excessive amount of protein, about 200 gm. of imidazole complex are excreted; hence about 40 per cent of this must have been exogenous in origin.

Fürth³ also observed a "Strenge Proportionalität zwischen Gesamtproteinumsatz und Diazochromogene;" but on the basis

of Masslow's work⁶ he concludes that the excretion of diazo chromogens is entirely independent of the protein content of the diet. We do not believe that Fürth's own work warrants such a conclusion because it shows a very definite relationship between diazo chromogens and protein intake. Neither he nor Masslow, however, have worked with a purified imidazole fraction. Their determinations were carried out on a crude urine containing phenols, aromatic hydroxy acids, urochrome, urochromogen, etc., which we can conclude from the fact that their only purification consisted in a precipitation of the urine with barium hydroxide. This may explain, in part, the contradictory results obtained by Masslow.

Excretion of Imidazoles under Pathological Conditions.

We now investigated the excretion of imidazoles under a variety of disease conditions. The material for this part of our work was furnished by the patients of the medical service of one of us (K) in the Cook County Hospital.

The diet which these patients received was either the regular ward diet or a soft diet. The regular ward diet contained about 1,925 calories which are furnished by 59.5 gm. of protein, 62.6 gm. of fat, and 263 gm. of carbohydrate. The soft diet contained 86.3 gm. of protein, 95.5 gm. of fat, and 330 gm. of carbohydrate which furnish about 2,592 calories. In certain ailments, such as gastric ulcer and nephritis without edema, a milk diet, consisting of 74 ounces of milk and 200 gm. of white bread, was given. This diet yields 1,852 calories and contains 81.7 gm. of protein, 99.3 gm. of fat, and 166 gm. of carbohydrate. Table II is a summary of our results on some of these cases showing the imidazole excretion in 24 hours.

While most of the patients consumed all the food brought to them, which was controlled by observation on the part of the nurses, there were some whose condition did not permit the intake of a liberal diet. This is true of patients who were in a highly febrile and toxic stage of acute and subacute infectious diseases such as pneumonia, mumps, miliary tuberculosis, etc. Some of the patients were in a state of partial or complete starvation either on account of inability to swallow because of mechanical obstruc-

TABLE II.
Urinary Imidazole Excretion under Pathological Conditions.

Case No.	Name.	Date.	Total volume per 24 hrs.	Imidazole value.	Clinical diagnosis.
		1980	cc.		
1	M. G.	Dec. 20	630	139	Lobar pneumonia before crisis.
		" 29	395	66	" " day of crisis.
		" 30	600	129	" " " after crisis.
2	E. D.	" 13	645	79	Carcinoma esophagi. Inanition.
		1981			
3	J. R.	Jan. 12	460	102	Pulmonary tuberculosis.
4	L. M.	" 19	810	219	Carcinoma of pancreas. Severe jaundice.
5	W. C.	" 6	910	134	Pancreatic cyst. Verified by operation.
6	J. S.	" 22	1,105	171	Endocarditis. Rheumatism. Arthritis.
7	R. L.	" 24	490	100	Mumps.
8	W. N.	" 12	1,354	255	Acute articular rheumatism.
9	O. S.	" 27	960	128	Aortic regurgitation. Arthritis.
10	H. L.	" 12	405	70	Carcinoma of stomach. Inanition.
11	P. G.	" 6	780	58	" " colon. Inanition.
					Senility (80 yrs.).
12	J. G.	" 22	285	34	Chronic alcoholism. Cirrhosis of liver. Ascites.
13	J. V.	" 27	1,025	151	Fibrocaceous tuberculosis pulmonum.
14	M. R.	Feb. 3	830	71	Carcinoma esophagi. Inanition.
15	J. G.	Apr. 18	930	138	Arthritis. Endocarditis.
16	A. N.	" 20	1,250	277	Acute catarrhal jaundice.
17	S. W.	May 25	1,840	185	Aneurysm of aorta. Emphysema.
		1988			
18	H. H.	Jan. 30	780	128	Arthritis. Pericarditis.
19	A. C.	Feb. 18	1,310	103	Diabetes mellitus.
20	C. B.	Mar. 7	835	86	Miliary tuberculosis. Insufficient nourishment.
21	D. H.	Apr. 11	990	139	Acute rheumatism.
22	W. A.	" 14	1,190	123	Plastic pleuritis.
23	W. G.	" 18	1,875	77	Lobar pneumonia. (Insufficient nourishment.)
24	M. K.	" 14	1,510	166	Acute rheumatic arthritis.
25	J. H.	" 18	855	159	Carcinoma of stomach with metastasis of liver.
26	E. K.	May 15	1,650	122	Carcinoma of stomach with huge metastasis in liver.

TABLE II—*Concluded.*

Case No.	Name.	Date.	Total volume per 24 hrs.	Imidazole value.	Clinical diagnosis.
		1922	cc.		
27	J. K.	May 16	2,600	107	Gastric ulcer. Milk diet.
28	H. B.	" 17	2,100	44	Acute rheumatic fever. No salicylates. <i>Nephritis?</i>
29	M. Z.	" 26	280	55	Inoperable carcinoma of stomach. Inanition.
30	A. H.	" 26	1,450	58	Splenic anemia. (Malaria?) Cachexia.
31	C. K.	" 29	1,750	129	Duodenal ulcer. Postoperative fibrous peritonitis. Lues hepatitis? Wasserman ++.
32	L. S.	" 29	1,110	132	Acute rheumatic fever. No salicylates.
33	J. F.	June 5	1,390	127	Gastric ulcer. Light diet.
34	S. J.	" 17	770	77	Lymphatic leucemia. Cyanosis. Aortic and mitral insufficiency. Albumin ++.
35	R. S.	" 18	2,000	123	Graves disease. Hyperthyroidism. Myocarditis.
36	P. D.	" 19	120	13	Huge inoperable carcinoma of stomach. Metastasis in liver. No food or water ingested.
37	W. N.	" 22	850	85	Carcinoma of stomach. Huge metastasis in liver.
38	O. B.	" 26	1,220	63	Chronic joint disease. Low protein cereal and vegetable diet.

tion of the digestive passages (esophagus or stomach) or because they were in a state of stupor or coma.

These patients, who were in a state of inanition, excreted abnormally small amounts of the imidazole complex.

One would expect to find that the excretion of imidazoles is to some extent a function of the water elimination. This is generally recognized as a fact for certain crystalloids, such as urea and uric acid, and is true even when the compounds in question are readily soluble in water. Any pathological condition that is associated with a retention of water by the tissues or in the large cavities of the body is always associated with a retention of some of the water-

soluble crystalloids. This is also true of the imidazole complex. Thus; *e.g.*, in Case 12 of our series (Table II) the imidazole value is 34 which means that only 34 mg. of imidazole complex were excreted in the urine in 24 hours. This patient had a chronic cirrhosis of the liver with an enormous ascites and the total amount of urine excreted in 24 hours was only 285 cc.

Patients afflicted with malignant tumors of the esophagus, stomach, or intestines (see Table II, Cases 2, 10, 11, 14, 29, 36, and 37) consistently excrete subnormal amounts of the imidazole complex. All these patients were in a state of inanition with marked emaciation and desiccation of the tissues. The volume of urine excreted in 24 hours was decidedly decreased. These patients had been undernourished for months and the low imidazole excretion is undoubtedly due, in part, to the small imidazole intake. There is, however, another factor that should be considered in this connection. Cancer is always associated with a toxic destruction of the body proteins. The protein catabolism is decidedly increased in carcinomatous patients so that even on ample nourishment a nitrogen equilibrium cannot be established. On first thought one would be tempted to conclude that the excretion of the imidazoles should be *increased* under these conditions because so large a part (60 per cent) of the imidazoles excreted *normally* appear to be endogenous in origin. Even in normal individuals, however, only a small fraction of the imidazoles ingested appear in the urine, which may mean that the larger part of the imidazole complex is disrupted (see introduction). In cancer, where the catabolism of proteins is so definitely increased, this disruption of the imidazole complex may be intensified so that the imidazoles are excreted in subnormal amounts. It is, of course, also possible that the relative imidazole excretion is, in fact, increased if the relation to the total nitrogen excretion is taken into consideration. We expect to report on this phase of the problem in a future publication.

Excretion of Imidazoles in Nephritis.

After establishing the values of the imidazole excretion for healthy individuals and in a large variety of diseases, we concentrated our attention on the excretion of the imidazole compounds in the different forms of kidney disease. It was in this phase of the

problem that we were, *a priori*, most interested; for the imidazole compounds are nitrogen-containing protein derivatives and the study of other nitrogenous catabolites such as urea, uric acid, and creatinine has yielded valuable information in studying the excretory function of the kidney. The newer detailed study of this function shows definitely that every substance, crystalloid or colloid, excreted by the kidney, follows its own laws which are determined by the relation of the substance to the tissues (extrarenal influences) and by its specific type of excretion through the renal parenchyma (renal factor).

We have studied the imidazole excretion, the concentration of other non-protein nitrogen constituents of the urine, the concentration of the corresponding non-protein nitrogen compounds in the blood, and the elimination of phenolsulfonephthalein, in the same patients and at the same period of the disease. In this way we have been able to compare the elimination of the imidazoles with the elimination and retention of the other normal nitrogenous catabolites. The phenolsulfonephthalein figures are of value because they afford a means for comparing the elimination of a substance entirely foreign to the organism with the excretion of a group of substances that constitute a normal catabolite of protein metabolism.

The experiments on normal individuals have demonstrated the important effect of diet on the imidazole excretion. As far as possible, the nephritics were kept on the standard "nephritic" diet of the Cook County Hospital which has the approximate composition shown in Table III.

Some of the patients, who were free from edema, were unable to eat the above solid diet. They were, therefore, given the diet that has been referred to on page 816 as the standard milk diet.

The results of these studies are summarized in Table IV which is arranged with special reference to the imidazole excretion. Those patients listed in the uppermost part of the table show a normal imidazole excretion. These are followed by patients who show a progressively decreasing elimination of the imidazole complex.

In discussing the results we wish to group the kidney disorders under three headings: namely, (1) Vascular renal sclerosis, which includes essential hypertension and interstitial nephritis (genuine

TABLE III.
Nephritic Diet.

Material.	Amount.	Protein.	Fat.	Carbohydrate.	Total calories.
Breakfast.					
Stewed fruit.					
Peaches.....	100 gm.	0.7	0.1	9.4	42.0
Oatmeal.....	150 "	4.2	0.7	17.2	94.5
Bread.....	50 "	4.6	0.6	26.5	134.0
Oleomargarine.....	10 "	0.12	8.3		77.7
Milk.....	150 cc.	4.9	6.0	7.5	108.0
Sugar.....	10 gm.			10.0	41.0
Total for breakfast.....		14.52	15.7	70.6	497.2
Dinner.					
Mashed potato.....	150 gm.	3.9	4.5	26.7	166.5
Carrots.....	100 "	1.1	0.4	9.3	46.0
Bread.....	50 "	4.6	0.6	26.5	134.0
Oleomargarine.....	10 "	0.12	8.3		77.7
Custard.					
Milk.....	100 cc.	3.3	4.0	5.0	72.0
Egg, $\frac{1}{2}$		2.2	1.7		26.5
Milk.....	150 "	4.9	6.0	7.5	108.0
Total for dinner.....		20.12	25.5	75.0	630.7
Supper.					
Creamed cabbage.					
Cabbage.....	50 gm.	0.8	0.1	2.8	16.0
Cream sauce.....	50 "	2.07	7.7	5.0	101.0
Bread.....	50 "	4.6	0.6	26.5	134.0
Oleomargarine.....	10 "	0.12	8.3		77.7
Rice custard.....	100 "	4.0	4.6	31.4	182.0
Stewed fruit					
Apple.....	100 "	0.2	0.8	37.2	161.0
Total for supper.....		11.79	22.1	102.9	671.7
Total for the day.....		46.43	63.3	248.5	1,799.6

TABLE
Urinary Excretion of

Case No.	Name.	Age.	Date.	Blood pressure.	Total volume per 24 hrs.	Specific gravity.	Total N.	Non-protein N.	Urea.	Uric acid.	Creatinine
							In the blood.				
				mm. Hg	cc.		mg.	mg.	mg.	mg.	mg.
1	J. T.	22	Mar. 14, 1922	S. 115* D. 75	850	1.018	2,884		29		1.5
2	J. B.	54	Jan. 17, 1921	S. 160 D. 100	785	1.015			47	4.8	
3	R. K.	75	Jan. 27, 1921	S. 260 D. 118	715	1.016	2,366	38	42	2.2	1.4
4	T. M.	70	May 16, 1922	S. 255 D. 120	2,050	1.010	2,525	30	23		1.4
5	J. K.	72	Jan. 25, 1921	S. 175 D. 115	1,740	1.009	2,291	31	37	2.7	1.3
6	P. V.	41	Apr. 23, 1921	S. 250 D. 130	1,180	1.006	2,650	26	46	4.1	1.6
7	C. B.	37	Nov. 23, 1921	S. 170 D. 70	3,000	1.011		25	26	2.0	1.1
8	R. C.	49	Jan. 26, 1922	S. 188 D. 130	1,580	1.022			80		2.1
9	S. S.	57	Feb. 8, 1922	S. 285 D. 145	1,375	1.012	2,520	40	40	6.6	1.3

* S represents systolic; D, diastolic.

IV.

Imidazoles in Nephritis.

Phenolsulfo- phthalein.	Imidazole index.	Clinical diagnosis.	Remarks.
<i>per cent excretion</i>	<i>mg.</i>		
1 hr. 50 2 hr. 10 60	209	Subacute glomerular nephritis. Bronchitis. Tonsillitis.	Albuminuria accidentally discovered. Several coarsely granular casts.
1 hr. 15 2 hrs. 25 40	155	Essential hypertension.	History of syphilis. Wassermann reaction —. Albumin absent. Occasional leucocytes and granular casts.
1 hr. 10 2 hrs. 5 15	140	Arteriosclerosis. Chronic interstitial nephritis. Cardiac hypertrophy?	Headaches; dizziness; ringing of ears; insomnia; loss of vision; swollen eyelids; senile emphysema Wassermann reaction —.
	100	Hypertension. Arteriosclerosis. Atheroma of aorta. Chronic interstitial nephritis.	Dyspnea; precordial pain and anxiety. Corrigan's pulse.
1 hr. 2 hr. 25 25	90	Essential hypertension. Arteriosclerosis. Chronic interstitial nephritis.	Albumin absent. Few hyaline and granular casts.
1 hr. 25 2 hrs. 20 45	86	Essential hypertension with secondary kidney involvement. Contracted kidney.	Albumin present. Few hyaline and granular casts.
1 hr. 20 2 hrs. 10 30	84	Acute exacerbation of chronic diffuse nephritis. Amyloid degeneration of kidneys.	Nocturia. Microscopic hematuria. Albumin in quantity. Lues. Wassermann reaction +. Edema of face and legs.
1 hr. 18 2 hrs. 10 28	77	Chronic glomerular nephritis. Myocarditis.	Albumin in quantity. Many coarse and fine granular casts.
1 hr. 25 2 hrs. 15 40	68	Genuine contracted kidneys. (Chronic interstitial nephritis.) Hypertension.	Hemiplegia 2 yrs. previously. Severe headaches. Albumin in quantity. Hyaline and fine granular casts.

TABI

Case No.	Name.	Age.	Date.	Blood pressure.	Total volume per 24 hrs.	Specific gravity.	Total N.	Non-protein N.	Urea.	Uric acid.	Creatinine.
							In the blood.				
				mm. Hg	cc.		mg.	mg.	mg.	mg.	mg.
10	C. H.	66	Feb. 11, 1921	S. 230 D. 120	1,030	1.020	2,791	28	28	4.6	1.
11	F. M.	48	Feb. 28, 1921	S. 148 D. 80	920	1.015	2,372	50	65	4.5	2.
12	M. F.	67	June 26, 1922	S. 185 D. 140	1,500	1.016	2,940		70		2
13	A. Z.	40	Mar. 9, 1921	S. 270 D. 170	560	1.018	3,682	32	34	6.1	1
14	C. B.	47	May 17, 1922	S. 150 D. 110	640	1.015	3,585	38	36	2.6	1
15	S. J.	28	Apr. 28, 1922	S. 140 D. 105	1,070	1.018	3,262	45	49		1
16	W. J.	38	June 26, 1922	S. 218 D. 170	1,570	1.010	2,450	50	105		1
17	G. N.	58	Jan. 14, 1920	S. 170 D. 80	2,460	1.012		38	36	3.5	1
18	F. R.	45	Apr. 4, 1922	S. 234 D. 140	1,530	1.012		61	85	4.4	

IV—Continued.

Phenolsulfophthalein.	Imidasole index.	Clinical diagnosis.	Remarks.
<i>per cent excretion</i>	<i>mg.</i>		
1 hr. 20	64	Arteriosclerosis. Chronic interstitial nephritis. Chronic arthritis.	Edema of ankles and right knee. No albumin. Several hyaline and granular casts. Few leucocytes and red cells.
2 hrs. 25			
45			
	60	Chronic glomerular nephritis.	Albumin in quantity. Many granular casts, leucocytes, erythrocytes, and epithelial cells. General edema.
1 hr. 10	57	Chronic glomerular nephritis with hypertension and edema.	Cardiovascular lues? Wassermann reaction —.
2 hrs. 10			
20			
	50	Chronic interstitial nephritis. Hypertension. Cardiac hypertrophy.	
1 hr. 5	48	Chronic diffuse glomerular nephritis.	Post mortem: subacute glomerular nephritis. Marked arteriosclerosis. Chronic passive congested kidney; hydroperitoneum; hydrothorax.
2 hrs. 10			
15			
	45	Secondary chronic diffuse nephritis or passive congestion of kidney.	Albumin in quantity. Numerous fatty and hyaline casts. A few granular casts.
1 hr. 10	45	Hypertensive nephritis without edema. (Genuine contracted kidney.)	
2 hrs. 0			
10			
1 hr. 10	44	Chronic glomerular nephritis. Aortic and mitral insufficiency. Luetic aortitis.	Albumin in quantity. General edema. Ascites. History of Scarlet fever and rheumatism.
2 hrs. 15			
25			
1 hr. 10	43	Interstitial nephritis. Genuine primary contracted kidney. Hypertension. Lead poisoning.	Anatomic diagnosis. Chronic nephritis. (Granular kidneys.)
2 hrs. 15			
25			

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T

Case No.	Name.	Age.	Date.	Blood pressure.	Total volume per 24 hrs.	Specific gravity.	Total N.	Non-protein N.	Urea.	Uric acid.
							In the blood.			
				mm. Hg	cc.		mg.	mg.	mg.	mg.
19	G. M.	27	Dec. 18, 1921	S. 135 D. 85	1,670	1.016			30	3.3
20	H. S.	36	June 16, 1922	S. 180 D. 115	520	1.020			87	
21	E. M.	51	Mar. 9, 1922	S. 120 D. 90	1,625	1.012			133	4.1
22	J. C.	55	Jan. 26, 1922	S. 255 D. 130	725	1.016		41	46	2.4
23	E. S.	39	Mar. 29, 1922	S. 205 D. 105	3,500	1.008	2,380		108	6.7
24	M. P.	71	Feb. 2, 1921	S. 180 D. 68	650	1.022	2,715	64	90	7.8
25	S. C.	36	Feb. 15, 1921	S. 100 D. 80	700	1.030	2,384	31	33	3.3
26	J. C.	25	Feb 21, 1922		875	1.028	3,525		22	3.5

IV—Continued.

Phenolsulfo- phthalein.	Imidazole index.	Clinical diagnosis.	Remarks.
<i>per cent excretion</i>	<i>mg.</i>		
1 hr. 60 2 hrs. 15 75	41	Passive congestion of kidney. Endocarditis. Mitral ste- nosis.	Albumin present. Blood and casts absent. Edema of eyelids, legs, and scrotum. Fibrillating heart.
1 hr. 40 2 hrs. 10 50	40	Subacute glomerular nephritis with hypertension. Edema.	Hydropericardium, hydroperi- toneum, and hydrothorax. Chlo- rides in the blood, 940 mg. per 100 cc.
1 hr. 5 2 hrs. 5 10	39	Chronic parenchymatous ne- phritis. (Chronic degener- ative epithelial nephrosis.)	Cholesterol 230 mg.
1 hr. 40 2 hrs. 10 50	36	Chronic interstitial nephritis with hypertension. Cardiac hypertrophy.	Chlorides 600 mg. Dyspnea on ex- cretion. Headaches. Nocturia. Loss of weight 50 lbs. Pain in joints. No edema. Albumin +. Hyaline and granular casts.
1 hr. 0 2 hrs. 5 5	34	Chronic glomerular nephritis. Contracted kidney. Chron- ic uremia. Hypertension.	Creatinine on Apr. 8, 1922 was 8.18 mg. per-100 cc. blood. Anatomic diagnosis. Chronic diffuse ne- phritis. Small granular kidneys.
1 hr. 20 2 hrs. 22 42	34	Chronic glomerular nephritis. Chronic myocarditis.	Anorexia. Edema of legs and eye- lids. Numerous granular casts. Epithelial cells, pus, and blood.
	32	Chronic parenchymatous ne- phritis. (Chronic degener- ative epithelial nephrosis.) Syphilis.	Albumin in quantity. Hyaline and granular casts. Edema. Bilateral hydrothorax. No edema at time of urine examination for imidazoles.
	30	Acute epithelial nephrosis. Bichloride poisoning.	Albumin slightly + only once. Blood absent. Mercury in stom- ach washings and urine. Hem- atemesis. Melena.

TABLE

Case No.	Name.	Age.	Date.	Blood pressure.	Total volume per 24 hrs.	Specific gravity.	Total N.	Non-protein N.	Urea.	Uric acid.	Creatinine.
							In the blood.				
				mm. Hg	cc.		mg.	mg.	mg.	mg.	mg.
27	J. J.	34	Dec. 13, 1921	S. 120 D. 90	625	1.021			21		1.5
28	T. M.	63	Mar. 15, 1922	S. 245 D. 90	1,150	1.015	2,870		43	4.1	1.5
29	V. A.	50	Nov. 3, 1921	S. 120 D. 75	930	1.012		29	27	2.0	1.6
30	M. G.	55	Jan. 11, 1921	S. 208 D. 112	775	1.010	2,130	76	114		3.3
31	H. B.	39	May 13, 1921	S. 288 D. 170	1,040	1.008	2,830		80	3.3	2.4
32	E. M.	27	Nov. 20, 1920	S. 200 D. 100	280	1.020		157	246	8.3	5.5

contracted kidney); (2) Chronic glomerular nephritis; and (3) Miscellaneous nephropathies.

1. Vascular Renal Sclerosis. (Including Essential Hypertension and Interstitial Nephritis (Genuine Contracted Kidney)).

The first and often only stage of this renal disease is known as "essential" hypertension. The kidney parenchyma proper in this condition often shows no microscopic changes at all, post mortem, or only such histological alterations in the vascular structure, glomeruli, and vasa afferentia as are found in the capillaries

IV—Concluded.

Phenolsulfo- phthalein.	Imidasole index.	Clinical diagnosis.	Remarks.
<i>per cent excretion</i>	<i>mg.</i>		
1 hr. 35 2 hrs. 20 55	27	Chronic diffuse nephritis. Polyserositis.	Anatomic diagnosis. Chronic dif- fuse nephritis. Pericarditis. Endocarditis. Pleuritis. Puru- lent peritonitis.
1 hr. 13 2 hrs. 7 20	26	Chronic interstitial nephritis Hypertension.	Dyspnea. Nocturia. Edema of face. Albumin in quantity. Granular and hyaline casts. Few red cells and pus cells.
	22	Bence-Jones proteinuria. Secondary chronic nephritis. Multiple myeloma.	Loss of weight 40 lbs. Myeloma or carcinoma of lumbar vertebræ.
1 hr. 2 2 hrs. 2 4	21	Chronic interstitial nephritis. Cirrhosis of liver. Lues.	Albumin in quantity. Esbach's reagent 0.40 per cent.
1 hr. 1 2 hrs. 2 3	18	Chronic diffuse nephritis Hy- pertension. Left cardiac hypertrophy.	Anatomic diagnosis. Chronic dif- fuse nephritis. Uremia. Spon- taneous intrapontine hemorrhage.
1 hr. 0 2 hrs. 0 0	0	Chronic glomerular nephritis. Uremia. Hypertension.	Albumin in quantity. Esbach's re- agent 7.5 p.m. Many granular and waxy casts.

and arterioles of other organs; i.e., hyaline degeneration of the vessel walls, hyperplastic thickening of the intima, and increase in the elastic fibers of the media. At this stage the disease is really not to be considered as a kidney affection proper, but as a disease of the general vascular system, usually associated with left cardiac hypertrophy. At first this condition is functional and transitory, undoubtedly due to arteriolar spasm. Later on, the increased muscular tonus of the vessel walls becomes permanent and with it also the hypertension (primary essential hypertension). The end-results of this condition are usually generalized arterial

fibrosis and sclerosis which frequently involves the kidneys leading ultimately to the changes known pathologically as contracted kidney. Clinically, however, one can readily distinguish two types of hypertensive nephrosclerosis which possess such definite characteristics that such a distinction seems justified, although, post mortem, the kidneys might show a similar or identical picture. The first type is a very chronic benign condition and, therefore, found frequently in middle age and more often in old people with definite external evidences of general vascular sclerosis. Renal compensation persists for a long time or until the end; for the atrophic changes in the secretory structures of the renal parenchyma, following the ischemia produced by the vascular sclerosis, progress very slowly and might exist for many years without any subjective symptoms. The objective signs on the part of the heart in response to the increased peripheral resistance and, not unlikely, to synchronous involvement of the myocardium by the same toxin, lead to a very strong diffuse apex impulse, left cardiac hypertrophy, enlargement of the aortic dullness, and accentuation of the second aortic tone. In some instances myocardial insufficiency with passive congestion of the lung and liver and general edema introduce the final stage of the disease. In most instances, however, such a stage does not develop and the patients grow to be old without any subjective or objective symptoms until cerebral hemorrhage leads to death. Some of these patients consult the physician frequently on account of subjective symptoms on the part of their cardiovascular system such as palpitations, headache, nocturnal paroxysmal attacks of dyspnea, or attacks of angina and nocturia. Renal signs are neither subjectively nor objectively predominant. albuminuria is either absent or only present to a slight degree, a few hyaline casts might be found, the ability to concentrate is usually well preserved, the NaCl excretion is not impaired, and the non-protein nitrogen constituents in the blood are scarcely, if at all, increased. The water test frequently shows deviations from the normal (latent nocturia). These patients seldom die a renal death; but the condition of their heart and blood vessels determines the duration of life. The great majority of them do not feel ill until they are felled suddenly by cerebral hemorrhage. The second type of patients who show, post mortem, a primary con-

tracted kidney, present a very different picture, clinically. These are relatively young people between the ages of 38 and 48, more frequently men than women. They are suddenly struck by a disease which affects all their mental and physical energies and in many respects suggests a subacute intoxication. The hypertension is usually above 200 mm. The cardiac hypertrophy is pronounced, the plethoric exterior is soon followed by an ashen grey color, and a severe anemia with marked decrease of hemoglobin and erythrocytes is frequently found though renal and extrarenal hemorrhages are absent. Slight edema of the face is frequently associated with this grey pallor and introduces the terrible picture which has been aptly designated as renal cachexia. This type of nephrosclerosis is commonly combined with a marked retention of nitrogenous catabolites in the blood; the kidneys have lost their faculty to concentrate and to dilute. This condition seldom lasts longer than 1 year and death results from toxic myocardial insufficiency, fatal hemorrhage, and not seldom with the picture of chronic uremia (malignant nephrosclerosis).

The first stage of renal sclerosis, essential hypertension, is represented by Case 2 of Table IV.

A more advanced stage of this same condition leading toward generalized arteriosclerosis and cardiac incompetency is illustrated by Cases 3 to 6. Patients represented by Cases 3, 4, and 5 were very old men (75, 70, and 72 years of age, respectively), while the last patient, Case 6, was only 41 years of age.

The end-state of vascular nephrosclerosis, chronic interstitial nephritis (genuine contracted kidney), is represented by Cases 9, 10, 13, 16, 18, 22, 28, and 30.

In reviewing the imidazole excretion in these thirteen cases of nephrosclerosis, the impression is gained that the determination of these compounds in a 24 hour specimen of urine is of decided value in estimating the excretory efficiency of the kidney in this condition. The benign forms of hypertension with just beginning renal decompensation show a slight decrease in the quantity of imidazoles excreted. The severer forms of arteriosclerotic nephritis with increasing involvement of the renal parenchyma show a more marked decrease and the severe forms of genuine contracted kidney are associated with an excretion of imidazoles as low as one-sixth of the normal value.

II. Chronic Glomerular Nephritis.

Eleven of our cases can be placed under this heading; namely, Nos. 8, 11, 12, 14, 17, 20, 24, 25, 27, 31, and 32. These cases vary in degree of severity and in time of duration. Some are associated with both edema and hypertension; some are associated with only one of these symptoms. A survey of Table IV shows that the imidazole excretion was decidedly decreased in every case. One of these patients—Case 32—did not excrete any imidazole compounds during a 24 hour period. The phthalein excretion was also nil. This patient died in uremic coma.

III. Miscellaneous Nephropathies.

Under this heading are comprised one case of acute focal glomerular nephritis, Case 1, one of amyloid degeneration of the kidney, Case 7, two of parenchymatous nephritis (chronic degenerative epithelial nephrosis), Cases 21 and 25, two of passive congestion of the kidney, Cases 15 and 19, one of acute epithelial tubular nephrosis due to bichloride poisoning, Case 26, and one case of Bence-Jones proteinuria with secondary chronic nephritis, Case 29.

CONCLUSIONS.

A survey of the imidazole excretion in 32 cases of nephritis of different types, given in Table IV, suggests that in general the excretion of imidazoles is inversely proportional to the severity of the disease. Cases of simple hypertension with little involvement of the kidney show an imidazole value that is either inside the normal limits or only slightly below these limits. The greater the involvement of the kidney parenchyma the greater the quantity of imidazoles retained in the body, or as we have determined it, the smaller the quantity of imidazoles excreted in the urine. The urinary excretion of imidazoles usually proceeds parallel with the excretion of the other nitrogenous catabolites. The latter are, however, usually determined in the blood and the retention of uric acid, urea, and creatinine in the blood is a valuable index for the decreased excretory function of the kidney. For the present it is not feasible to determine the imidazoles in the blood or plasma; but in the majority of cases where the concentration of the other nitrogenous catabolites in the blood is *increased*, the concentration

of imidazoles in the urine is correspondingly *decreased*. An *absolute* parallelism between the retention of any of the other nitrogenous catabolites—uric acid, urea, and creatinine—and the imidazole complex seems not to exist. Some cases that show a greatly diminished excretion of imidazoles give a normal blood value for urea (Cases 26, 27, and 29) or a normal blood value for creatinine (Cases 25, 26, 27, 28, and 29). This lack of definite correlation emphasizes anew the fact that *each metabolite follows its own laws regarding its retention in the tissue and excretion through the kidneys*.

A comparison of the imidazole excretion with the phenol-sulfonephthalein excretion generally also reveals a certain parallelism. Thus the severest degrees of disturbance in the renal function as measured by the phthalein excretion correspond to the ones in which the imidazole excretion was impaired to the greatest degree (Cases 23, 30, 31, and 32).

SUMMARY.

1. When urine, normal or pathological, is treated with lead acetate and sodium hydroxide in the proper proportions, a precipitate is obtained that contains, besides other substances, the aromatic hydroxy acids, the polyphenols, and all the urinary coloring matter.

2. The water-clear filtrate from the above lead precipitate contains all of the imidazoles.

3. The imidazoles can be most conveniently determined in the above lead-free filtrate by means of the colorimetric process, previously described by us, which is based upon the reaction that occurs between imidazoles and *p*-phenyldiazonium sulfonate in alkaline (Na_2CO_3) solution.

4. By means of this simple, rapid method, the imidazole excretion in the urine has been quantitatively followed in normal individuals in a variety of diseases, and particularly in nephritis.

5. A 24 hour specimen of urine obtained from a healthy, normal adult contains from 120 to 220 mg. of imidazole complexes.

6. The excreted imidazoles are chiefly endogenous in origin. To a variable extent, however, the excretion of imidazoles is de-

pendent upon the protein content of the diet. Thus in one case, 118 mg. of imidazole complex were excreted on a low protein diet and 172 mg. on a high protein diet.

7. In pathological conditions the imidazole value has been found to vary from 0 to 277 mg.

8. Patients suffering from nephritis, who showed a nitrogen retention in the blood, gave the lowest imidazole values. The excretion of imidazoles was, in general, inversely proportional to the severity of the disease.

9. An imidazole excretion of less than 80 mg. on an adequate diet seems to indicate a definite impairment of the excretory function of the kidney. Values below 40 mg. suggest a very unfavorable state of the secreting renal tissue in the sense of a partial loss of function through destruction.

10. The determination of the imidazoles in the urine promises to yield valuable information regarding the protein catabolism in disease and regarding the excretory function of the kidney.

STUDIES ON PROTEINOGENOUS AMINES.

XVII. ON THE FACULTY OF NORMAL INTESTINAL BACTERIA TO FORM TOXIC AMINES.

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INTRODUCTION.

In a series of papers published in 1919¹ we communicated a method for the microchemical colorimetric estimation of imidazole derivatives and for the quantitative separation of histamine from histidine. These methods have enabled us to study the metabolism of histidine under a variety of conditions and by a large number of microorganisms. Our previous work led us to conclusions that can be summarized briefly as follows:

1. Of the organisms studied so far, only those belonging to the colon group—in the narrower sense, which includes *Bacillus coli communis*, *Bacillus coli communior*, *Bacillus lactis aerogenes*, and *Bacillus acidi lactici*—have been able to convert histidine into histamine in a synthetic medium.

2. Of the twenty-nine strains of coli that we studied, six were able to decarboxylate histidine.

3. This decarboxylation of histidine occurs only when the medium contains an available source of N—ammonia or potassium nitrate—and an available source of carbon—glycerol or glucose—along with the histidine. In the absence of an available nitrogen source, histidine remains either unchanged or is deaminized. In the absence of an available carbon source, the histidine is disrupted, the imidazole complex being destroyed.

* We have been assisted, in the bacteriological work, by Dr. Jennie Ada Walker.

¹ Koessler, K. K., and Hanke, M. T., *J. Biol. Chem.*, 1919, xxxix, 497, 521.

4. The addition of either leucine, alanine, arginine, glycine, or peptone to the standard synthetic medium augments the growth of the colon bacillus, and increases the yield of histamine. When glutamic acid or tryptophane are added to the standard medium, the growth of the microorganisms is augmented; but the quantity of histamine formed is decreased. Cystine is unfavorable to the growth of the colon bacillus. The presence of this amino acid reduces the yield of histamine to almost nil.

Up to the time of our last publication,² we had studied the metabolic action of microorganisms when grown in pure culture in a synthetic medium containing histidine. The present publication is a summary of the results obtained: (a) with naturally occurring mixtures of microorganisms, particularly those native to the large intestines of man; and (b) with pure cultures of bacteria isolated from such mixtures and grown on synthetic media containing as the only amino acids present either histidine or tyrosine.

EXPERIMENTAL.

Procedure.

The mixture of microorganisms, or pure strains obtained from such mixtures (the exact quantities are given later in the paper) were introduced into 200 cc. of an autoclaved medium having the following composition.

0.2000	gm. amino acid (histidine dichloride or tyrosine).
0.2000	ammonium chloride.
0.1000	potassium nitrate.
0.4000	“ dihydrogen phosphate.
0.8000	sodium chloride.
0.0200	“ sulfate (anhydrous).
0.4000	“ bicarbonate.
0.0100	calcium chloride (anhydrous).
4.00	cc. glycerol.

These were dissolved in sufficient distilled water to give a final volume of 200 cc.

The inoculated media were then incubated at 37°C. for 14 days unless otherwise specified. The remainder of the procedure varied, according to the amino acid employed, as follows:

² Hanke, M. T., and Koessler, K. K., *J. Biol. Chem.*, 1922, 1, 131.

Histidine.

The bacterial mixture was forced through a Berkefeld filter by suction. The hydrogen ion concentration of the filtrate was determined colorimetrically. The filter was then carefully washed with at least 200 cc. of distilled water. Concentrated H_2SO_4 —1.0 cc.—was added to the combined filtrate and washings, which were then freed from water by evaporation in a glass dish on the water bath. The syrupy residue was transferred, with distilled water, to a 25 cc. precision cylinder and diluted to exactly 25 cc. Of this test liquid 10 cc. were transferred to a 35 cc. glass-stoppered bottle, treated with 3 gm. of solid NaOH , and extracted six times with amyl alcohol, using 20 cc. for each extraction. This divides the material into two fractions, the amyl alcohol extract, which may contain histamine and methyl imidazole and which we refer to as the histamine fraction, and the alkaline aqueous liquid which contains the unchanged histidine and which may contain imidazole acetic, -propionic, -lactic, and -acrylic acids.

The combined amyl alcohol extracts were extracted with N H_2SO_4 , which removes the imidazoles. The acid extracts were nearly neutralized with 5 N NaOH and the resulting liquid was diluted to 100 cc. Chloroform—10 cc.—was added to the contents of the cylinder and the mixture agitated to remove the excess of amyl alcohol. The amount of histamine present was then determined colorimetrically by means of the well known reaction that occurs between *p*-phenyldiazonium sulfonate and imidazole derivatives in a solution rendered alkaline with sodium carbonate.¹ When the presence of histamine was indicated by the colorimetric determination, an amino nitrogen determination was also carried out, of which, however, no detailed account is given subsequently because the agreement between the colorimetric values and the amino nitrogen values was always very good.

The histidine fraction was transferred to a 25 cc. graduated precision cylinder with water and 7 cc. of 37 per cent HCl . The cooled acid liquid was then diluted to 25 cc. Chloroform—10 cc.—was added to remove the amyl alcohol. Of this histidine fraction, 5 cc. (measured by pipette) were transferred to a 100 cc. graduated cylinder and diluted to 80 cc. Colorimetric determinations were carried out on 0.10 to 0.50 cc. portions of this diluted solution. In every case an amino nitrogen determination

was carried out on the histidine fraction. In cases where the imidazole complex had not been appreciably disrupted by the microorganisms, the amino nitrogen figure agreed very well with the colorimetric value. When the colorimetric results indicated a disruption of the imidazole complex, the amino nitrogen figure was always higher than the colorimetric value. Our report contains only the colorimetric values, which we know to be a true index of the amount of histidine present. None of these mixtures of microorganisms gave results that would lead us to expect the presence of imidazole acetic, -propionic, -lactic, or -acrylic acids.

Tyrosine.

Estimation of Volatile Phenols.—The bacterial mixture was treated with 0.5 cc. of 95 per cent H_2SO_4 and transferred, with water, to a 1,000 cc. long-necked, round-bottom Pyrex flask. The flask was heated by means of a gas burner through a small hole in an asbestos gauze. The hot vapors were condensed in a spiral condenser, the distillate being collected in a 100 cc. graduated cylinder. Exactly 100 cc. of distillate were collected. This distillate was then tested immediately for phenols, using the method previously described.³ Volatile phenols were not produced in any of our experiments.

The liquid left in the flask was transferred, with water, to a glass dish and concentrated on the water bath. The residue, which always contained dead bacteria and coagulated protein, was transferred, with water, to a 25 cc. precision cylinder and diluted to 25 cc. This mixture was then filtered, through a small, dry filter paper, into a dry, glass stoppered bottle. This is the test liquid. It is brown in color, but clear. The filtration is not time-consuming in spite of the mass of coagulated protein, etc.

Estimation of Aromatic Hydroxy Acids.—Of this acid test liquid, 10 cc. (measured by pipette) were transferred to a 35 cc. extraction bottle and extracted ten times with specially prepared ether.³ The ether extracts were treated with 25 cc. of water and 5 drops of 85 per cent H_3PO_4 , the mixture was agitated and sub-

³ The procedure used in estimating the individual phenols is reported in the *J. Biol. Chem.*, 1922, 1, 235. A method for the quantitative separation and subsequent estimation of phenols is reported in the *J. Biol. Chem.*, 1922, 1, 271.

jected to a distillation at first under ordinary pressure and then *in vacuo* until the ether had been removed entirely. An ebullition tube was not used and the distillation was not continued until all the water had passed over. The aqueous liquid left in the flask was carefully transferred, with water, to a 100 cc. graduated cylinder and diluted to 100 cc. The concentration of aromatic hydroxy acids was then determined, colorimetrically, using the method previously described.³ Color values were, in every case, calculated as hydroxyphenyllactic acid because the time of evolution of the color and its stability warranted the conclusion that this acid was present.

Separation of Tyramine from Tyrosine.—The acid liquid left in the extraction bottle, equivalent to 10 cc. of the test liquid, was treated with 3 gm. of solid sodium hydroxide. The mixture was cooled under the tap while the hydroxide was dissolving. The resulting strongly alkaline solution was then extracted eight times with amyl alcohol, using 20 cc. for each extraction.

The Alkaline Aqueous Liquid (Tyrosine Fraction).—The alkaline aqueous liquid left in the extraction bottle was transferred to a glass dish with water and 7 cc. of 37 per cent HCl. The resulting acid solution was concentrated on the water bath. This removes the amyl alcohol completely. The residue was transferred, with water, to a 25 cc. graduated cylinder and diluted to exactly 25 cc. Of this solution, 5 cc. (measured by pipette) were diluted to exactly 80 cc. The colorimetric estimation of tyrosine was carried out on 0.10 and 0.20 cc. portions of this solution, using the method previously described.³ An amino nitrogen determination was also carried out in all cases on 2 cc. portions of the undiluted tyrosine fraction. The amino nitrogen figure was always considerably higher than the colorimetrically determined tyrosine value. This is easily understood when we consider that the microorganisms were not removed in this case by Berkefeld filtration, but were heated for some time in a strongly acid solution which must, of course, have dissolved considerable of their nitrogenous constituents. The latter would then enhance the amino nitrogen figure.

Determination of Ammonia in the Histidine and Tyrosine Media.

Of the original *test liquid*, total volume 25 cc. (see pages 838 and 839), 5 cc. were treated with 5 gm. of solid K_2CO_3 and aerated for 1 hour, using the apparatus described by Van Slyke and Cullen.⁴ The ammonia was collected in 10 cc. of 0.1 N HCl. The excess of acid was then determined by titration with 0.1 N NaOH using sodium alizarin sulfonate as indicator.

The ammonia originally introduced into the synthetic media as hydrochloride would neutralize 36.4 cc. of 0.1 N HCl. When the tabular values are higher than this, ammonia was produced by the microorganisms from the amino acids, histidine or tyrosine. When these values are lower than 36.4 cc., which is usually the case, ammonia was consumed by the micro organisms. In most cases the figures indicate that the organisms assimilated both ammonia nitrogen and amino acid nitrogen.

The Amyl Alcohol Extract (Tyramine Fraction).—The combined amyl alcohol extracts were extracted five times with N H_2SO_4 , using 20 cc. for the first and 10 cc. for each of the remaining four extracts. Tyramine passes quantitatively into the acid solution. The combined acid extracts were transferred to a glass dish, neutralized with 5 N NaOH, and treated with 2 gm. of solid sodium carbonate. The liquid was concentrated somewhat on the water bath. This removes the ammonia completely. The alkaline liquid was now treated with 5 cc. of 37 per cent HCl and concentrated on the water bath until the excess of HCl had been removed. The crystalline residue was dissolved in water, transferred to a 25 cc. precision cylinder, and diluted to 25 cc. Of this solution, 5 cc. (measured by pipette) were diluted to exactly 80 cc. The colorimetric estimation of tyramine was carried out on 0.10 to 0.50 cc. portions of this solution, using the method previously described.³

An amino nitrogen determination was also carried out in all cases on 2 cc. portions of the undiluted tyramine fraction. The figure obtained agreed fairly well in most cases with the colorimetrically determined values of tyramine.

⁴ Van Slyke, D. D., and Cullen, G. E., *J. Biol. Chem.*, 1914, xix, 218.

TABLE I.

Catabolism of Histidine and Tyrosine by the Mixture of Micro Organisms Contained in Human Feces.

No.	Name.	Unchanged histidine.		Reaction of histidine medium after incubation. Initial pH 7.3.	Unchanged tyrosine.		Tyrosine converted into aromatic hydroxy acids. (Hydroxy-phenyl-lactic acid.)	Reaction of tyrosine medium after incubation. Initial pH 7.3.	0.1 N HCl neutralised by NH ₃ from the entire test solution.	
		per cent	per cent	pH	per cent	per cent	per cent	pH	Histidine. cc.	Tyrosine. cc.
1	E. H.	92	0	5.2	4.38	95.6	0	5.2	44.0	20.0
2	C. C.	69	25	5.2	1.25	90.0	7.5	5.2		
3	O. H.	16	7.7	5.2						
4	J. W.	10	51.6	5.0	56.0	18.0	0	5.2	12.0	34.0
5	M. M.	10	44.0	4.8	7.0	86.0	0	5.2	15.0	32.5
6	H. H.	6	28.0	5.2	12.8	87.5	0	5.0	40.0	32.0
7	M. H.	12	43.0	4.8	13.0	70.0	0	5.0	16.0	34.0
8	L. B.	8	59.0	4.8	9.0	78.0	0	5.0	17.0	35.0
9	J. R.	15	44.0	5.2	75.0	13.5	9.0	5.0	19.0	35.0
10	D. C.	40	25.0	5.2	30.0	50.0	9.0	5.2	12.0	17.5
11	L. S.	15	25.0	5.0	20.0	65.0	0	5.0	19.0	32.5
12	H. S.	50	2.0	5.0	6.0	78.0	0	5.2	39.6	38.0
13	K. F.	33	14.5	5.0	7.0	78.0	0	4.8	41.0	29.0
14	M. S.	65	0 0	5.0	81.0	0	0	5.2		35.0
15	J. L.	56	16.0	5.0	81.0	0	0	5.2	35.0	36.0
16	S. S.	71	4.6	5.0	75.0	0	0	5.0	34.0	38.0
17	M. L.	68	12.4	5.2	80.0	0	0	5.2	34.0	34.0
18	L. C.	83	0	5.2	78.0	0	0	5.2	33.0	35.0
19	H. E.	65	0	5.4	90.0	0	0	5.2		
20	B. F.	79	0	5.0	21.0	78.0	0	5.4	36.0	41.0
21	No. 37	63	0	5.4	56.0	20.0	0	5.4	33.0	29.3
22	" 89	0	56	5.4	20.0	32.5	0	6.2	18.3	35.0
23	" 10	63	0	5.2	82.0	0	0	5.0	34.0	31.0
24	" 34	35	0	5.0	70.0	12.0	0	5.0	32.5	36.7
25	" 17	72	0	5.2	70.0	12.0	0	5.2	32.5	36.0
26	" 25	10	0	5.6	0	0	0	5.0	21.3	5.2

I. Catabolism of Histidine and Tyrosine by the Mixture of Microorganisms Contained in Human Feces.

Of the stool, which was collected with as little contamination as possible, about 10 c. mm. were introduced into 200 cc. of our synthetic medium. The mixtures were incubated at 37°C. for 14 days. They were then subjected to analysis, using the methods that have just been described. The growth was luxuriant in every case.

Table I is a summary of our results on twenty-six samples of whole stool, eighteen of which were obtained from perfectly healthy, normal men and women, the remaining eight having been obtained from cases as follows:

Case 1.—E. H. Chronic enteritis. Arthritis deformans. This patient has been afflicted for the last 12 years with a chronic progressive inflammation of practically all the joints of the body. Extensive degenerative changes have led to partial ankylosis of both knee joints and a very extensive spondylitis resulting in a coalescence of all cervical vertebræ. There seemed to exist, during the whole course of his disease, a rather definite relationship between intestinal upsets—consisting of gastric distress, complete loss of appetite, and diarrhea—and the relapses of his joint involvement. Thus, the last attack during Oct., 1922, beginning as usual with the gastrointestinal symptoms described above, resulted in the new involvement of his mandibular joint leading to an almost complete inability to separate his jaws.

Case 2.—Chas. C. Age 7 years. The boy has suffered from intestinal disturbances (diarrhea, green stools) since he was 1 year old. He has a history of measles, at the age of 2, followed by "uremia" of 11 months duration interspersed with severe bronchial colds. The bronchial condition was not relieved by tonsilectomy. At the age of 4 he had the "flu" associated with double pneumonia and followed by "uremia." Since then he has been afflicted with a persistent dyspnea. His mother had the impression that severe attacks of bronchial colds and dyspnea were always preceded by gastrointestinal disturbances. He has had an evening temperature during the past 3 or 4 years. At the time he was examined by one of us (K), his temperature was 98.6, white blood count 18,600, eosinophils none. Sensitization tests for foods, animal inhalants, pollen, and bacteria were negative. There was a certain opacity in the right maxillary sinus with questionable ethmoid involvement on both sides. X-rays of the lungs showed marked infiltration in both hila and extensive fibrosis and chronic inflammation of a non-tuberculous type. The sputum contained *Staphylococcus albus*, *Streptococci (viridans and hemolyticus)*, and *B. fusiformis*. There seemed to be a definite relationship between the intestinal upsets and his attacks

of bronchiolar spasm and, therefore, a careful bacteriological study of his stools seemed advisable.

Case 21.—Patient 37. Acute articular rheumatism.

Case 22.—Patient 89. Lobar pneumonia.

Case 23.—Patient 10. Subacute endocarditis.

Case 24.—Patient 34. Atrophic cirrhosis of liver.

Case 25.—Patient 17. Hodgkin's disease.

Case 26.—Patient 25. Subacute rheumatism.

A survey of the table shows:

1. Of the 26 stools, 16 (62 per cent) contained organisms that were able to decarboxylate histidine, 17 (65 per cent) contained organisms that were able to decarboxylate tyrosine and, of these, 12 (46 per cent) contained organisms that were able to decarboxylate *both* histidine and tyrosine.

2. Of the strictly normal stools—18 in all—14 (78 per cent) contained organisms capable of decarboxylating histidine, 11 (61 per cent) contained organisms capable of decarboxylating tyrosine and, of these, 10 (56 per cent) contained organisms capable of decarboxylating *both* histidine and tyrosine.

3. Volatile phenols were never produced, from tyrosine, in a synthetic medium that contained carbohydrate.

4. Hydroxyphenyllactic acid was produced, from tyrosine, in only 3 of the 26 cases and then only in small amounts (7.5 to 9 per cent conversion).

5. The pH dropped uniformly from 7.3 to 5.2 to 5.0. The mixture of microorganisms produced more acid than did the majority of pure strains of colon bacilli previously studied by us.

II. Catabolism of Histidine and Tyrosine by Pure Strains of Microorganisms Isolated from Human Feces.

Having shown that the faculty of decarboxylating histidine and tyrosine is a common property of the normal inhabitants of the intestinal tract of man, we were now interested in the identity of the microorganisms that were responsible for this activity. Two of the samples of feces were, therefore, subjected to the customary bacteriological analysis. This yielded 11 strains of microorganisms, in pure culture, from the Chas. C. stool and 8 strains, in pure culture, from the E. H. stool. Table II is a classification chart of these 19 strains of microorganisms. The metabolism of

TABLE II.—*Differential Characteristic*

Derivation of strain.	No. of strain.	Motility.	Gram stain.	Colony on endo.	Glycerol agar.		Liquefaction of gelatin.	Coagulation of milk.		Dextrose.	Lactose.		Dulcitol.	Saccharose.		Mannitol.	
					Growth.	Gas.		Gas.	pH.		Gas.	pH.		Gas.	pH.		
Chas. C.	Organism 1	+	-	Red.	+	+	+	+	+	5.2	+	5.2	+	5.2	+	5.2	+
	" 2	+	-	"	+	+	-	+	+	5.2	+	5.2	+	5.0	+	5.0	+
	" 3	+	-	"	+	+	-	+	+	5.2	+	5.0	+	5.2	+	5.0	+
	" 4	+	-	"	+	+	-	+	+	5.2	+	5.2	+	5.0	+	5.0	+
	" 5	+	-	"	+	+	-	+	+	5.0	+	5.0	+	5.2	+	5.0	+
	" 7	*	-	"	+	-	-	+	+	5.0	+	5.0	-	7.6	+	5.0	+
	" 8	+	-	"	+	+	-	+	+	5.0	+	5.0	+	5.2	+	5.0	+
	" 9	+	-	"	+	+	-	+	+	5.0	+	5.0	+	5.0	+	5.0	+
	E. H.	" 1	+	-	"	+	+	+	+	+	5.6 ±	5.4	-	7.6	+	5.2	+
" 2		+	-	"	+	+	-	+	+	5.2	+	5.2	+	5.0	-	7.0	+
" 3		-	+	"	+	+	-	+	+	5.2	+	-	+	+	+	+	+
" 4		+	-	"	+	+	-	+	+	5.2	+	5.2	+	5.0	-	6.4	+
" 6		+	+	"	+	-	+	+	+	5.2	+	5.2	+	7.6	+	7.0	+
" 7		-	+	"	+	-	-	+	-	5.2	-	6.4	-	6.4	-	6.4	-
" 8		-	+	"	+	-	-	+	-	5.2	-	5.0	-	6.4	-	5.0	-
" 9		-	+	"	+	-	-	-	-	+	-	-	-	-	-	-	-
Cheese.		No. B	-	+	Red.	+	-	-	+	+	6.0	-	6.4	-	6.6	-	6.4

*Sluggish.

histidine and tyrosine in our synthetic medium by these organisms is summarized in Tables III and IV. A survey of these tables shows the following:

1. Histamine producers were not among the organisms isolated.

2. Seven of the eleven organisms isolated from the stool of Chas. C. converted tyrosine into tyramine. These Gram-negative organisms were members of the colon-typhoid group. They have retained their decarboxylase activity for 1 year, grown on glycerol agar.

3. Two of the nine organisms isolated from the stool of E. H. converted tyrosine into tyramine. They are Gram-positive,

Microorganisms Studied.

Rhamnose.	Salicin.		Maltose.		Galactose.		Xylose.		Levulose.		Arabinose.		Inulin.		Indole.	Classification.
pH	Gas.	pH	Gas.	pH	Gas.	pH	Gas.	pH	Gas.	pH	Gas.	pH	Gas.	pH		
5.2	+	5.2	+	5.0	+	5.0	+	5.2	+	5.2	+	5.0	-	7.6	-	<i>B. coli communior.</i>
5.2	+	5.0	+	5.2	+	5.0	+	5.2	+	5.0	+	5.0	-	7.6	-	" " "
5.2	+	5.2	+	5.0	+	5.0	+	5.2	+	5.0	+	5.0	-	7.6	-	" " "
5.0	+	5.0	+	5.0	+	5.0	+	5.0	+	5.0	+	5.0	+	5.8	+	" " "
5.2	+	5.0	+	5.0	+	5.0	+	5.0	+	5.2	+	5.2	-	7.6	+	" " "
5.0	-	5.0	+	5.0	+	5.0	+	5.0	+	5.0	+	5.0	-	7.6	+	<i>B. lactis aerogenes.</i>
5.2	+	5.0	+	5.0	+	5.0	+	5.0	+	5.0	+	5.0	+	7.2	-	<i>B. coli communior.</i>
5.0	+	5.2	+	5.0	+	5.0	+	5.0	+	5.0	+	5.0	+	7.6	+	" " "
7.6	-	7.6	+	5.4	+	5.0	+	5.0	-	5.8	-	7.6	±	7.6	-	<i>B. pyocyaneus.</i>
6.4	±	6.6	+	5.2	+	5.2	+	5.0	+	5.2	+	5.0	±	6.8	+	<i>B. coli communior.</i>
±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	-	<i>Staphylococcus.</i>
7.2	+	7.6	+	5.2	+	5.0	+	5.0	+	5.0	+	5.2	±	7.6	+	<i>B. coli communior.</i>
7.6	±	5.4	+	5.2	-	7.6	-	6.4	-	5.0	-	7.3	±	7.6	-	Gram-positive, spore-forming aerobic (unidentified).
6.4	-	5.2	-	5.4	-	7.6	-	7.6	-	5.2	-	6.4	-	7.6	-	<i>B. acidophilus.</i>
6.4	-	5.2	-	4.8	-	4.8	-	6.4	-	4.8	-	6.4	-	6.4	-	" " "
±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	-	<i>Staphylococcus (?)</i> .
6.4	-	5.8	-	6.0	-	6.4	-	6.4	-	6.2	-	6.2	-	5.6	-	<i>B. acidophilus.</i>

belong to the acidophilus group and may be two strains of the same organism. After growing for 1 year on glycerol agar, they have lost their faculty to decarboxylate tyrosine.

III. Catabolism of Histidine and Tyrosine by the Organisms Contained in Swiss Cheese.

The experiments on Swiss cheese were conducted before we had demonstrated the fact that microorganisms, that had the faculty of decarboxylating tyrosine, could be so readily obtained from normal human feces. After we had completed the method for the separation and colorimetric estimation of tyrosine and its derivatives, we sought for an organism that would catabolize tyrosine to convince ourselves that the method, which gave such

TABLE III.
Catabolism of Histidine and Tyrosine by Microorganisms Isolated from the Stool of Chas. C.

Laboratory No.	Classification.	Unchanged histidine.		Histidine converted into histamine.		Reaction of histidine medium after incubation for 14 days. Initial pH 7.5.		Time of incubation. Unchanged tyrosine.		Time of incubation. Tyrosine converted into tyramine.		Time of incubation. Tyrosine converted into hydroxyphenyl-lactic acid.		Reaction of tyrosine medium after an incubation period of:		0.1 N HCl neutralised by NH ₃ from the entire test solution.	
		per cent	per cent	per cent	per cent	pH	per cent	14 days.	40 days.	per cent	14 days.	40 days.	per cent	14 days.	40 days.	cc.	cc.
1	<i>B. coli communior.</i>	90	0	0	95	5.2	9.0	0	0	0	0	0	5.0	6.2	38.5	28.0	22.0
2	" "	85	0	21	98	5.2	0	0	0	81	0	0	5.2	5.2	38.0	25.0	13.0
3	" "	90	0	75.0	0	5.2	75.0	0	0	14	0	0	5.4	5.2	38.0	23.0	26.0
4	" "	75	0	77.0	0	5.2	77.0	0	0	0	0	0	5.2	5.0	36.0	36.0	38.0
5	" "	83	0	19.0	81	5.2	19.0	0	0	0	0	0	5.2	6.0	35.0	29.0	21.0
6	Gram-negative bacillus not identified.	82	0	92.0	0	5.2	92.0	83.0	0	0	0	0	5.4	5.2	37.0		34.0
7	<i>B. lactis aerogenes.</i>	77	0	28.0	75	5.2	28.0	28.0	0	75	0	0	5.2	5.2	36.0	34.0	32.0
8	<i>B. coli communior.</i>	75	0	14.0	87	5.2	14.0	0	0	95	0	0	5.2	5.2	35.0	38.0	28.0
9	" "	77	0	9.0	81	5.0	9.0	9.0	0	81	9.25	9.25	5.2	5.2	37.5	27.0	27.0
10	Gram-negative bacillus not identified.	60	0	97.0	0	5.2	97.0	97.0	0	0	0	0	5.6	5.6	36.0	35.0	
11	" "	87	0	94.0	0	5.2	94.0	81.0	0	0	0	0	5.2	5.2	36.0	31.0	

TABLE IV.
Catabolism of Histidine and Tyrosine by Microorganisms Isolated from the Stool of E. H.

Laboratory No.	Classification.	Unchanged histidine.		Histidine converted into histamine.		Reaction of histidine medium after incubation for 14 days. Initial pH 7.3.		Time of incubation. Unchanged tyrosine.		Time of incubation. Tyrosine converted into tyramine.		Time of incubation. Tyrosine converted into hydroxyphenyl-lactic acid.		Reaction of tyrosine medium after an incubation period of:		0.1 N HCl neutralised by NH ₃ from the entire test solution.			
		per cent	per cent	per cent	per cent	pH	pH	14 days.	40 days.	14 days.	40 days.	14 days.	40 days.	pH	pH	Histamine. 14 days.	Tyrosine. 14 days.	cc.	cc.
1	<i>B. pyocyaneus.</i>	67	0	5.2	62.5	95.0	0	0	0	0	0	0	0	4.8	4.8	38.0	8.5	2.75	
2	<i>B. coli communis.</i>	75	0	5.2	94.0	95.0	0	0	0	0	0	0	0	5.2	5.2	35.2	34.5	37.5	
3	<i>Staphylococcus.</i>	71	0	5.4	83.0	80.0	0	0	0	0	0	0	0	5.4	5.4	27.5	30.0	29.75	
4	<i>B. coli communis.</i>	63	0	5.6	85.0	85.0	0	0	0	0	0	0	0	5.2	5.2	30.0	40.5	39.4	
6	Gram-positive spore-forming aerobe not identified.	0	0	6.4	65.6	40.0	0	0	0	0	0	0	0	5.8	6.0	43.0	35.0	40.0	
7	<i>B. acidophilus.</i>	66	0	5.2	72.0	70.0	22.5	30.0	0	0	0	0	0	5.4	5.4	36.7		39.0	
8	" "	80	0	5.0	55.5	61.0	45.0	42.0	0	0	0	0	0	5.4	5.2	36.5	38.6	39.0	
9	<i>Staphylococcus</i> (?).	70	0	6.0	64.5	53.0	0	0	0	0	0	0	0	5.8	5.8	16.0	33.0	31.5	

TABLE V.
Catabolism of Histidine and Tyrosine by Microorganisms Contained in Swiss Cheese.

Laboratory No.	Classification.	Unchanged histidine.	Histidine converted into histamine.	Reaction of histidine medium after incubation for 14 days.		Time of incubation. Tyrosine unchanged.		Time of incubation. Tyrosine converted into tyramine.		Time of incubation. Tyrosine converted into hydroxy-phenyl-lactic acid.		Reaction of tyrosine medium after an incubation period of:		0.1 N HCl neutralized by NH ₃ from the entire test solution.
		per cent	per cent	pH	per cent	14 days	40 days	per cent	14 days	40 days	per cent	14 days	40 days	
	Inoculated with 100 c.m.m. of whole cheese.	88	0	5.0										
A	<i>Staphylococcus</i> (Gram-positive).					64	0	0	0	0	*	6.2	*	*
B	<i>Bacillus acidophilus</i> .					11.5	0	87.5	95	0	0	5.2	*	31
														6.0
														25.0

* Not determined.

reliable results with mixtures of pure phenols, was equally applicable to the analysis of catabolism mixtures. In 1909, Winterstein and K \ddot{u} ng⁵ were able to isolate tyramine from Emmenthal cheese, and in 1914 Ehrlich and Lange⁶ isolated organisms that decarboxylated tyrosine. The work of these investigators indicated that Swiss cheese was a promising source from which to isolate an organism that possessed a decarboxylase activity for tyrosine. We, therefore, inoculated portions of our synthetic medium at first with whole cheese (about 100 c. mm.) and subsequently with the organisms isolated, in pure culture, from the cheese. Table V is a summary of the results obtained. The cultural characteristics of the two organisms isolated from this sample of cheese are included in Table II.

The mixture of organisms contained in cheese did not decarboxylate histidine. The enzymatic activity was so intense, in the tyrosine medium, that all the tyrosine was converted into tyramine. This included not only that introduced as free tyrosine but also that contained in the cheese.

The bacteriological analysis of the cheese led to the isolation of two microorganisms, in pure culture, that we have numbered A and B. Organism A, a staphylococcus, did not convert tyrosine into tyramine. Organism B, a member of the acidophilus group, converted 87.5 per cent of the tyrosine into tyramine in 14 days and effected a complete conversion in 40 days without attacking the tyramine. After growing for 1 year on glycerol agar, this organisms had lost its faculty for decarboxylating tyrosine.

This organism did not catabolize histidine.

DISCUSSION.

A study of the decarboxylase activity, toward histidine and tyrosine, of the mixture of microorganisms contained in human feces has shown that of the 26 stools investigated, 16 (62 per cent) decarboxylated histidine, 17 (65 per cent) decarboxylated tyrosine, and 12 (46 per cent) produced both histamine and tyramine.

A bacteriological analysis was conducted on two of the stool

⁵ Winterstein, E., and K \ddot{u} ng, A., *Z. physiol. Chem.*, 1909, lix, 138.

⁶ Ehrlich, F., and Lange, F., *Biochem. Z.*, 1914, lxiii, 156.

samples. One of the specimens (Chas. C.) yielded 11 strains of microorganisms; the other (E. H.) yielded 9 strains. Some of these, *e.g.* Nos. 10 and 11 of the Chas. C. stool and Nos. 7 and 8 of the E. H. sample, may be identical.

Although the mixture of microorganisms represented by the Chas. C. stool converted histidine into histamine, none of the organisms isolated from this mixture, had that faculty. One might venture to conclude, on the first aspect, that the amine production was the result of symbiotic influences. When, however, we consider the fact that pure strains of *Bacillus coli* have previously been isolated which decarboxylate histidine in a medium of identical composition, and that of 29 strains examined only 6 have this faculty, we are probably justified in concluding that the organism or organisms responsible for this enzymatic activity, escaped isolation.

In a subsequent paper, experiments are described which demonstrate clearly that *not only are amine-producing microorganisms constant inhabitants of the normal alimentary tract, but histamine appears also to be a constant constituent of cecal content and of fecal matter.*

Seven of the eleven organisms isolated from the stool of Chas. C. converted tyrosine into tyramine during a 2 week incubation period. Four of these organisms (Nos. 2, 7, 8, and 9 of Table III) did not catabolize tyramine; the quantity of amine produced during a 40 day incubation period was almost identical with that produced in 14 days. Organisms 1 and 5 show a very different behavior. After converting practically all the tyrosine into tyramine in the course of 14 days, these microorganisms now attacked the *tyramine* and catabolized it in such a manner that it had disappeared completely at the end of 40 days. The decarboxylase activity of Organism 3 came into action very slowly. Tyramine was not produced during a 14 day incubation period, but was present, to a small extent, after 40 days of incubation. The production of hydroxyphenyllactic acid was observed in only one case. This type of enzymatic activity appears, from our accumulated data, to be rare, at least in a medium containing carbohydrate.

The above cases are of interest, moreover, because they illustrate how differently identical species of bacteria may catabolize

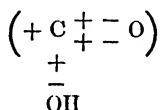
the same amino acid. The organisms are all strains of *Bacillus coli communior* isolated from the same specimen of stool. Their morphological, biological, and commonly studied chemical characteristics, as given in Table II, are almost identical; but the first forms 95 per cent of tyramine in 2 weeks and catabolizes it so completely in 40 days that it is no longer demonstrable; the second converts tyrosine into tyramine almost quantitatively in 2 weeks, but does not destroy it to any appreciable extent during 4 subsequent weeks; the third forms no tyramine after 14 days, but does so after 40 days; and the fourth does not catabolize tyrosine to tyramine, but does produce a small amount of hydroxyphenyllactic acid.

Of the nine organisms isolated from the E. H. stool, only two decarboxylated tyrosine. They belong to the acidophilus group. Their enzymatic activity was not very intense and suggested that the organisms were already losing or just acquiring the faculty of decarboxylation. Subsequent work has shown that these organisms had lost their faculty for decarboxylation completely after growing for 1 year on glycerol agar. That organisms of the acidophilus group may produce amines, has not, heretofore, been known. We have isolated three organisms, two from the E. H. stool and one from cheese that belong in this group and that were active decarboxylators when freshly isolated. All these organisms lost their decarboxylase activity after they had been grown on artificial media for 1 year. This explains the fact that none of the stock strains of *Bacillus acidophilus*⁷ obtained by us from various laboratories would decarboxylate tyrosine.

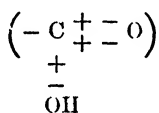
One fact that stands out prominently not only in Section II of this paper but also in some of our previous work, is the *specificity* of the decarboxylase activity. *Microorganisms that produce histamine do not decarboxylate tyrosine and tyramine producers do not decarboxylate histidine.* We are tempted, at this time, to attribute this specificity to a difference in the electronic constitution of the side chain in histidine and tyrosine and more particularly to a difference in the polarity of the charge with which the carboxyl group is bound to the side chain. We have

⁷ Some of these strains were obtained from Dr. L. F. Rettger, Professor of Bacteriology, Yale University, and we take this opportunity to thank him for these cultures.

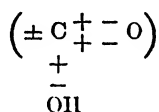
previously shown⁸ how the electronic constitution of a large number of organic compounds can be derived from their known chemical properties. The amino acids, however, because of their stability give us but little clue as to their intraatomic structure. From the electronic view-point it is possible to consider the amino acids as belonging to three distinct groups, if we take into consideration the charges of the carbon atom of the carboxyl group; namely, those having a distinctly positive carboxyl group



those having a distinctly negative carboxyl group



and those having an electrically uncertain carboxyl group



Of these, the amino acids having a distinctly positive carboxyl group may be decarboxylated by one group of organisms and those having a distinctly negative carboxyl group may be decarboxylated by a different group of organisms. It seems, just now, as if histidine might belong to the negative and tyrosine to the positive type. Experiments are now under way in this laboratory that may shed light on this obscure field of biochemistry.

SUMMARY.

1. A study of the decarboxylase activity toward histidine and tyrosine, of the mixture of microorganisms contained in human feces, has shown that of the 26 stools investigated, 16 (62 per cent)

⁸ Hanke, M. T., and Koessler, K. K., *J. Am. Chem. Soc.*, 1918, xl, 1726; *J. Biol. Chem.*, 1922, l, 193.

decarboxylated histidine, 17 (65 per cent) decarboxylated tyrosine, and 12 (46 per cent) produced both histamine and tyramine. A synthetic medium containing salts, glycerol, and histidine or tyrosine was inoculated with a small fragment of the total stool.

2. 18 of the 26 stools were obtained from normal individuals. Of these, 14 (78 per cent) contained microorganisms that decarboxylated histidine, 11 (61 per cent) decarboxylated tyrosine, and 10 (56 per cent) contained organisms that produced both histamine and tyramine.

3. Volatile phenols were never produced, from tyrosine, in our synthetic medium containing carbohydrate.

4. Hydroxyphenyllactic acid was produced, from tyrosine, in only 3 of the 26 cases and then only in small amounts (7.5 to 9.0 per cent).

5. The pH dropped uniformly from 7.3 to 5.2 to 5.0.

6. Two of the stools that had been found to contain amine producers were subjected to bacteriological analysis. In this way, 11 strains were isolated from the one stool and 9 strains from the other.

7. Histamine producers were not among the organisms isolated.

8. 7 of the 11 organisms isolated from the one stool converted tyrosine into tyramine. These Gram-negative microorganisms are members of the colon-typhoid group. They have retained their decarboxylase activity for at least 1 year, grown on glycerol agar.

9. 2 of the 9 organisms isolated from the other stool converted tyrosine into tyramine. These Gram-positive organisms were members of the acidophilus group. They have lost their faculty to decarboxylate tyrosine after 1 year of growth on glycerol agar.

10. A Gram-positive organism, also a member of the acidophilus group, that converted tyrosine into tyramine, was isolated from cheese. This organism has lost its decarboxylase activity after 1 year of growth on glycerol agar.

STUDIES ON PROTEINOGENOUS AMINES.
XVIII. ON THE PRODUCTION OF HISTAMINE, TYRAMINE, AND
PHENOL IN COMMON LABORATORY MEDIA BY
CERTAIN INTESTINAL MICROORGANISMS.

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INTRODUCTION.

During the past 6 years we have developed methods that enable us to determine toxic amines, particularly histamine, in protein-containing mixtures, and to follow the metabolic activities of microorganisms in culture media containing histidine and tyrosine.

Our bacterial metabolism experiments have, up to the present time, been limited to a study of the changes that occur in a synthetic medium consisting of salts, ammonia, and nitrate as sources of nitrogen, glycerol as a source of carbon, and the amino acids whose metabolism we wished to study, usually histidine or tyrosine. Such simple media are not, of course, representative of the conditions existing in and associated with the mammalian organism and we have been fully cognizant¹ of the fact that we must, ultimately, develop methods that could be applied to media that more closely approach naturally occurring conditions. The media finally selected were milk, with and without the addition of histidine or tyrosine, and peptone broth to which 5 per cent of either ascitic fluid or blood had been added, with and without the addition of histidine or tyrosine.

We have been gratified to find that the methods used on the simple synthetic media are applicable, with but few minor changes, to these more complex media.

* We have been assisted, in the bacteriological work, by Dr. Jennie Ada Walker.

¹ Hanke, M. T., and Koessler, K. K., *J. Biol. Chem.*, 1922, 1, 176.

During the past few years we have isolated a number of microorganisms that possess the ability to decarboxylate histidine or tyrosine. Our first series of experiments were carried out with typical representatives of these two classes of organisms; namely, "Coli cystitis," a *Bacillus coli communis*, our classical histamine producer, and C.S. 2, a *Bacillus coli communior* that has the faculty of decarboxylating tyrosine to tyramine.

Procedure.

The Media.—Three basic media were employed, namely ascitic fluid broth, which consisted of 190 cc. of peptone broth and 10 cc. of ascitic fluid, blood broth, composed of 190 cc. of peptone broth and 10 cc. of sheep blood, and milk, of which 200 cc. were used, after it had been carefully skimmed. The microorganisms were grown in each of these media with and without the addition of tyrosine or histidine dichloride, as specified below.

The Microorganisms.—The two microorganisms selected for this work were "Coli cystitis," a *Bacillus coli communis* that has shown a constant faculty for converting histidine into histamine^{2, 3} and C.S. 2, a *Bacillus coli communior* that has the faculty of converting tyrosine into tyramine.⁴ Of these organisms, 9 billion were separately introduced into 200 cc. of each of the media contained in 300 cc. Pyrex flasks. The mixtures were incubated for 14 days at 37°C.

Analytical Procedure.—These media all contained some histidine and tyrosine as constituents of the protein even when these amino acids were not deliberately added. It was necessary, therefore, to plan a mode of procedure that would be as instructive as possible with regard to the metabolism of each of these amino acids without being too time-consuming.

The milk medium differed from the others in that it contained a copious coagulum after incubation. This was removed by gravity filtration. The coagulum was triturated repeatedly with small portions of water to remove all extractable matter. Beyond this stage the mode of procedure was uniform for all of the media; namely, as follows:

² Koessler, K. K., and Hanke, M. T., *J. Biol. Chem.*, 1919, xxxix, 539.

³ Hanke, M. T., and Koessler, K. K., *J. Biol. Chem.*, 1922, l, 131.

⁴ See the preceding article.

The pH was determined colorimetrically³ on 1 cc. of the media. In the case of milk, this determination was carried out before the coagulum was removed by filtration.

The mixtures were separately transferred to a 1,000 cc. round-bottom, long-necked Pyrex flask, treated with 0.50 cc. of 95 per cent H_2SO_4 and subjected to a distillation under atmospheric pressure to remove phenol.^{5,6} Phenol was determined in the distillates as previously described.^{5,6} The residue left in the distilling flask contained a large amount of coagulated protein which was removed by gravity filtration. The filtrate and washings were transferred to a glass dish and evaporated on the water bath. The residue frequently contained so much coagulated protein that a refiltration was necessary. The residues finally obtained, nearly free from protein, were transferred, with water, to a 25 cc. graduated cylinder and diluted to 25 cc. The mixtures were filtered and the clear but highly colored test liquids were used for the subsequent determinations.

Of the clear test liquid, 10 cc. were measured, by pipette, into a 35 cc. extraction bottle.⁷ The *acid* liquid was then extracted ten times with specially prepared ether to remove aromatic hydroxy acids.⁶ The latter were determined in the ether extract as previously described.^{5,6}

The aqueous residue from the ether extracts was warmed to 60°C. for 1 hour to remove the ether. Solid NaOH —3 gm.—was then added. The *alkaline* liquid was extracted six times with amyl alcohol^{6,7} which removes histamine and tyramine. In our experiments only *one* of these amines was present at any time; so a special method for separating these amines was not needed. This separation can, when necessary, be very easily and quantitatively accomplished by means of a silver precipitation.⁸

The amines, histamine or tyramine, were removed from the amyl alcohol by means of $\text{N H}_2\text{SO}_4$. The concentration of amines was then determined colorimetrically in this acid extract, as previously described.⁷

⁵ Hanke, M. T., and Koessler, K. K., *J. Biol. Chem.*, 1922, 1, 235.

⁶ Hanke, M. T., and Koessler, K. K., *J. Biol. Chem.*, 1922, 1, 271.

⁷ The apparatus used in this extraction work and a detailed description of the mode of procedure are given in *J. Biol. Chem.*, 1919, xxxix, 521.

⁸ Hanke, M. T., and Koessler, K. K., *J. Biol. Chem.*, 1922, 1, 188.

The alkaline residues from the amyl alcohol extraction were transferred to a glass dish, with water. 7 cc. of 37 per cent HCl were added, which is sufficient to render the liquid slightly acid. The resulting solutions were evaporated to remove amyl alcohol, transferred to a 25 cc. graduated cylinder, and diluted to 25 cc.

The amino acid fraction was always rather heterogeneous. The amino acid concentration was high, as can be seen from the following figures.

Milk Medium.—0.50 cc. of the amino acid fraction gave, on the average, 1.90 cc. of N_2 at 20° and 750 mm.

Ascitic Fluid Broth.—0.50 cc. of the amino acid fraction gave 2.40 cc. of N_2 at 20° and 750 mm.

Blood Broth.—0.50 cc. of the amino acid fraction gave 2.50 cc. of N_2 at 20° and 750 mm.

The presence of peptone, and the mutual presence of histidine and tyrosine, made it impossible for us to determine the exact concentration of either histidine or tyrosine with a simple colorimetric process. The isolation and estimation of histidine could have been carried out; but the process is time-consuming and the exact results so obtained would have been of too little significance to warrant the expenditure of time. This fraction was, therefore, subjected to qualitative tests only. The diazo test was used for the determination of histidine. The Millon reaction was used for the determination of tyrosine.

Production of Tyramine and Phenol by Bacillus Coli Communior
CS. 2.

This is one of the organisms isolated from the stool of Chas. C. that had the faculty of converting tyrosine into tyramine, but not histidine into histamine, on a synthetic medium in which a strong acid reaction was developed by the catabolism of glycerol. The results obtained when this organism is grown on the common laboratory media are summarized in Table I. A survey of the table shows the following.

1. Tyramine or histamine were not produced in a medium that contained no carbohydrate and in which an acid reaction was not developed. In the milk medium, considerable acidity was developed and, in this case, tyramine was produced when tyrosine was

available. The milk medium to which tyrosine had not been added contained no available tyrosine and the production of tyramine was, of course, impossible in this case. The above results lend support to our previously published contention that *the production of amines from amino acids, by microorganisms, is a protective mechanism and is resorted to when the accumulation of H ions within the organism's protoplasm threatens to destroy its life.*

2. The peptone broth media that had been augmented by the addition of either blood or ascitic fluid, remained alkaline throughout the course of the experiment. In all of these cases phenol was produced. In those media to which tyrosine had not been added, the average production of phenol was 0.0105 gm. regardless of whether blood or ascitic fluid had been used along with the peptone broth. When tyrosine was added to the broth media, a total of about 0.0900 gm. of phenol was produced; 75 per cent of the tyrosine added had been converted into phenol. The other 25 per cent disappeared without leaving a clue as to its metabolism. Some of this was probably used for synthetic purposes and some may have adhered to the bulky protein precipitates.

Just a word should be said, at this time, about the method used in identifying phenol. Our standard colorimetric process, which is based upon the well known reaction that occurs when phenols or imidazoles are allowed to react with *p*-phenyldiazonium sulfonate in alkaline solution, can be used not only to estimate the amount of substance present but is also frequently a reliable method for determining the character of the compound. We have discussed this matter in detail in our previous publications,^{5,9} but wish again to call attention to the fact that phenol and *o*-cresol are the only substances we have studied that give an intensely yellow color.¹⁰ *p*-Cresol gives a red color. The distillates all gave the rapidly developing yellow color that is so characteristic of phenols whose *p*-position is not substituted. The production of *m*-cresol was hardly probable. To prove our case irrefutably we prepared the tribrom derivatives from some of the distillates and found that the resulting tribrom-phenol weighed approxi-

⁹ Koessler, K. K., and Hanke, M. T., *J. Biol. Chem.*, 1919, xxxix, 497.

¹⁰ In general, phenols in which the position para to the OH group is not occupied, should give yellow colors.

TABLE I.
Catabolism of Tyrosine in Some Common Laboratory Media Incubated 14 Days at 37°C.*

Experiment No.	Composition of the media.	Tyrosine converted into tyramine.	Tyrosine converted into aromatic hydroxy acids.	Tyrosine converted into phenol (C_6H_5OH).	Reaction of the medium.		0.1 N HCl neutralized by NH_3 from the entire solution.	Unchanged tyrosine.	Remarks.
					pH before incubation.	pH after incubation.			
1	Peptone broth... 190 cc. Ascitic fluid..... 10 "	None.	None.	0.01125	7.6	7.6	66.5	None.	Reaction was alkaline throughout. Phenol was produced. Tyramine and histamine were not produced. Histidine was not catabolized.
2	Same as No. 1 but sterilized at 15 lbs. pressure for 15 min.	None.	None.	0.0105	7.6	7.6	57.5	None.	The results were identical with those obtained in Experiment 1. Catabolism unchanged by heat sterilization of the initial medium.
3	Peptone broth... 190 cc. Ascitic fluid..... 10 " Tyrosine..... 0.2000 gm.	None.	None.	0.0900 76	7.6	7.6	72.5	None.	Reaction was alkaline throughout. Phenol was the only catabolism product of tyrosine. Histidine was not catabolized.

4	Peptone broth... Blood.....	100 cc. 10 "	None.	None.	0 0105	7.2	62.0	None.	Reaction was alkaline throughout. Substitution of blood for ascitic fluid produced no change in the metabolism results. Phenol was the only catabolism product of tyrosine. Histidine was not catabolized.
5	Peptone broth... Blood..... Tyrosine.....	100 cc. 10 " 0.2000 gm.	None.	None.	0 0867 74	7.2	123.5	None.	
6	Milk..... Heat sterilized.	200 cc.	None.	None.	None.	5.0	7.5	None.	Reaction acid after incubation. Tyrosine, tyramine, histamine, and Phenol absent. Histidine present.
7	Milk..... Tyrosine.....	200 cc. 0.2000 gm.	0.1000 gm. as tyramine hydrochloride. 52%	None.	None.	5.0	2.3	Tyrosine present.	Reaction acid after incubation. 52 per cent of tyrosine converted into tyramine. Phenol absent. Histamine absent.

* These studies were conducted with our stock CS. 2 organism, a *B. coli communior*.

TABLE II.
Catabolism of Histidine in Some Common Laboratory Media* Incubated 14 days at 37°C.

Experiment No.	Composition of the media.	Histidine converted into histamine.	Histidine converted into imidazoles other than histamine.	Reaction of the medium.			0.1 N HCl neutralized by NH ₃ from the entire test solution.	Unchanged histidine.	Remarks.
				Before incubation.	After incubation.	After incubation.			
1	Peptone broth.....	None.	None.	7.6	7.6	7.6	cc. 25.0	Present.	Reaction was alkaline throughout. Histamine absent. Phenol absent. Histidine and tyrosine present.
	Ascitic fluid.....								
2	Peptone broth.....	None.	None.	7.6	7.6	7.6	65.0	Present in quantity.	Same as No. 1. Histidine was not catabolized in this medium.
	Ascitic fluid.....								
	Histidine dichloride... 0.2000 gm.								
3	Peptone broth.....	None.	None.			7.3	37.0	Present.	The results were identical with those obtained in Experiments 1 and 2. The substitution of blood for ascitic fluid produced no change in the metabolism results.
	Blood.....								
4	Peptone broth.....	None.	None.			7.3	45.0	Present in quantity.	
	Blood.....								
	Histidine dichloride... 0.2000 gm.								

5	Milk Heat sterilized.	200 cc.	None.	None.	5.0	None.	None.	Reaction was acid after incubation. Histamine not produced because histidine was not present in available form.
6	Milk Histidine dichloride ...	200 cc. 0.2000 gm.	0.02342 gm. histamine dichloride 14.5%	None.	5.0	None.	Present in quantity.	Reaction was acid after incubation. 14.5 per cent of the histidine that was added, was converted into histamine.

* These studies were conducted with our stock "Coli cystitis" organism, a *B. coli communis*.

mately what we had calculated it should from the colorimetric determinations and that the melting point agreed perfectly with the recorded value (94–95°C.). A mixture of our tribromide with some tribromide prepared from pure phenol also melted at 94–95°C.

This most interesting phenomena, the production of phenol, is the subject of our next paper and we shall consider it in detail at that time.

3. This organism did not catabolize histidine.

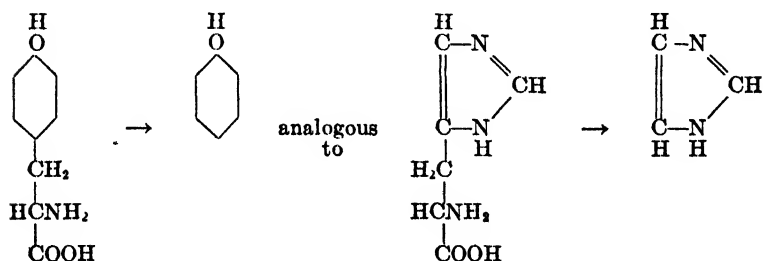
Production of Histamine by Bacillus Coli Communis (Cystitis).

"Coli cystitis," one of the stock strains with which we have worked since 1917, has the faculty of decarboxylating histidine to histamine in a synthetic salt medium in which an acid reaction has developed from the catabolism of glycerol or carbohydrate. This microorganism does not catabolize tyrosine.

The results obtained when this organism is grown on the common laboratory media are summarized in Table II. A survey of the table shows the following.

1. Histamine or tyramine was not produced in a medium that contained no carbohydrate and in which an acid reaction was not developed. Considerable acidity was developed in the milk media and, in this case, histamine was produced when histidine was present. Milk, *per se*, contains no available histidine.

2. The peptone broth media remained alkaline throughout the course of the experiment. Histidine appears not to have been attacked in any of these media. Imidazole, which could by analogy have been expected to occur as a catabolism product of histidine, was not produced.



3. Phenol was not produced in any of the media.

The two organisms used, both colon bacilli, differ sharply in that the one decarboxylates tyrosine and the other histidine. Either of the decarboxylase activities are called into play only when the medium acquires a strong acid reaction. The specificity of these two organisms is still more strikingly revealed by the fact that the organism that has the faculty of decarboxylating tyrosine to tyramine in acid solution also has the faculty of catabolizing tyrosine to phenol in alkaline solution. This organism does not catabolize histidine. The organism that converts histidine into histamine in acid solution does not catabolize histidine to imidazole and does not catabolize tyrosine at all.

The complete absence of *p*-cresol is rather surprising when we consider that the chief volatile phenol constituent of the urine of man and the herbivorous animals is *p*-cresol. Microorganisms producing *p*-cresol have, however, been isolated¹¹ and it is possible that its formation may be a faculty of still another group of microorganisms or, that suitable conditions for its production did not exist in our experiments. A detailed discussion in which all these facts are correlated, will be found at the end of the following paper.

SUMMARY.

1. Metabolism experiments have been conducted with two strains of colon bacilli on the common laboratory media, milk, blood broth, and ascitic fluid broth.

2. The colon bacilli used were special strains, one of which had been found to have the faculty of converting tyrosine into tyramine and the other of converting histidine into histamine.

3. Tyramine or histamine was not produced in a medium that contained no carbohydrate and in which an acid reaction was not developed. Considerable acidity was developed in the milk media and, in this case, histamine was produced by the one organism and tyramine by the other when the amino acids from which these amines are derived were added in an available form. These results support our previously published contention *that the production of amines from amino acids by microorganisms seems to be a protective mechanism and is resorted to when the accumulation of H*

¹¹ Baumann, E., and Preusse, C., *Z. physiol. Chem.*, 1880, iv, 455.

ions within the organism's protoplasm is incompatible with its normal life processes. The amines can be thought of as reaction buffers.

4. The blood broth and ascitic fluid broth media remained alkaline throughout the incubation period. The organism that has the faculty of decarboxylating tyrosine in an acid medium produced phenol from tyrosine in these alkaline media. This organism did not catabolize histidine. The microorganism producing histamine did not catabolize either histidine or tyrosine in the alkaline broth media. Phenol was not produced.

STUDIES ON PROTEINOGENOUS AMINES.

XIX. ON THE FACTORS INVOLVED IN THE PRODUCTION OF PHENOL BY THE COLON GROUP.*

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(Received for publication, January 26, 1924.)

INTRODUCTION.

The preceding paper contains a report of a number of metabolism experiments that were conducted on common laboratory media, with two strains of colon bacilli. One of these organisms had the faculty of decarboxylating tyrosine to tyramine in a synthetic salt medium and of catabolizing tyrosine to phenol in an alkaline ascitic fluid broth medium. This organism did not catabolize histidine. The other organism decarboxylated histidine to histamine in a synthetic salt medium, but did not catabolize tyrosine in any of the media.

These results suggested the following inquiries, which we shall attempt to answer in this communication.

1. Will any of the members of the colon group that have the faculty of decarboxylating tyrosine, in a medium in which an acid reaction is developed, catabolize tyrosine to phenol in an alkaline ascitic fluid broth medium?

2. Do any of the microorganisms that decarboxylate histidine to histamine, in a medium in which an acid reaction is developed, have the faculty of catabolizing tyrosine to phenol in an alkaline ascitic fluid broth medium?

* Throughout this article, when we refer to microorganisms of the colon group we restrict this term to include only *B. coli communis*, *B. coli communior*, *B. lactis aerogenes*, and *B. acidi lactici*.

† We have been assisted, in the bacteriological work, by Dr. Jennie Ada Walker.

3. Do any of that large group of "neutral" coli, those that decarboxylate neither histidine nor tyrosine, have the faculty of converting tyrosine into phenol?

4. What are the characteristics of a medium that determine whether or not phenol will be produced?

Mode of Procedure.

The Media.—A heat-sterilized ascitic fluid broth medium composed of 190 cc. of peptone broth, 10 cc. of ascitic fluid, and 0.2000 gm. of tyrosine was used in the comparative experiments by means of which an answer to the first three queries was obtained. In the last group of experiments, a variety of media was employed, fully described in Table II.

The Microorganisms.—Three groups of microorganisms were employed: namely, those that decarboxylated tyrosine in a synthetic salt medium (Coli 'S. 1, 2, 3, 5, and 8), those that decarboxylated histidine in a synthetic salt medium (Coli K (red) cystitis, Y, bovis 3, and P-5-19), and those that do not decarboxylate either histidine or tyrosine, of which we selected Coli E. H. 2, 4, and 5, and 'S. 4 and 9.

Analytical Procedure.—This was identical in every respect with that used and described in the preceding communication.

I. Colon Bacilli That Have the Faculty of Decarboxylating Tyrosine in Acid Media, Catabolize Tyrosine to Phenol in an Alkaline Broth Medium.

Up to the present time we have isolated five strains of colon bacilli that have the faculty of decarboxylating tyrosine in an acid medium. The results obtained when these organisms were grown on an ascitic fluid broth medium are summarized in Table I, Section I. A survey of the table shows the following.

1. Every one of the five strains of colon bacilli converted tyrosine into phenol. The percentage of conversion varied from 62.5 to 87. A reexamination showed that all these organisms had retained, unimpaired, their decarboxylase activity toward tyrosine in an acid medium.

2. Aromatic hydroxy acids and tyramine were not produced in any case.

TABLE I.
Catabolism of Tyrosine by Microorganisms of The Colon Group.

Experiment No.	Microorganisms used.	Composition of the medium.	Reaction of the medium.		0.1 N HCl neutralized by NH ₃ from the entire test solution.	Tyrosine converted into phenol (C ₆ H ₅ OH). Phenol, gm. Tyrosine converted, per cent.
			Before incubation.	After incubation.		

Section I.

					cc.	
1	CS. 1 <i>B. coli communior.</i>	Peptone broth... 190 cc. Ascitic fluid..... 10 " Tyrosine..... 0.2000 gm.	7.6	7.6	72.5	0.085 82
2	CS. 2 <i>B. coli communior.</i>	Same as No. 1.	7.6	7.6	72.5	0.090 87
3	CS. 3 <i>B. coli communior.</i>	Same as No. 1.	7.6	7.6	67.5	0.065 62.5
4	CS. 5 <i>B. coli communior.</i>	Same as No. 1.	7.6	7.6	60.0	0.090 87
5	CS. 8 <i>B. coli communior.</i>	Same as No. 1.	7.6	7.6	111.7	0.0725 70

Section II.

6	Cystitis. <i>B. coli communis.</i>	Same as No. 1.	7.6	7.6	55.5	None.
7	K(red) <i>B. coli communior.</i>	Same as No. 1.	7.6	7.6	83.0	None.
8	P-5-19 <i>B. acidi lactici.</i>	Same as No. 1.	7.6	7.6	69.0	None.
9	Y <i>B. coli communior.</i>	Same as No. 1.	7.6	7.6	55.4	None.
10	Bovis 3. <i>B. lactis aerogenes.</i>	Same as No. 1.	7.6	7.6	63.0	None.

TABLE I—Concluded.

Experiment No.	Microorganisms used.	Composition of the medium.	Reaction of the medium.		0.1 N HCl neutralized by NH ₃ from the entire test solution.	Tyrosine converted into phenol (C ₆ H ₅ OH). Phenol, gm. Tyrosine converted, per cent.
			Before incubation.	After incubation.		
Section III.						
11	E. H. 2 <i>B. coli communis.</i>	Same as No. 1.	7.6	7.6	63.0	None.
12	E. H. 4 <i>B. coli communis.</i>	Same as No. 1.	7.6	7.6	66.0	None.
13	E. H. 5	Same as No. 1.	7.6	7.6	110.7	None.
14	CS. 4 <i>B. coli communior.</i>	Same as No. 1.	7.6	7.6	50.4	0.035 34
15	CS. 9 <i>B. coli communior.</i>	Same as No. 1.	7.6	7.6	61.6	None.

3. The reaction of the medium remained alkaline throughout.

From these experiments it is evident that the faculty of converting tyrosine into tyramine in acid media and that of catabolizing tyrosine to phenol in alkaline media, are closely related enzymatic activities.

A colon bacillus that has the faculty of decarboxylating tyrosine in acid media, catabolizes tyrosine to phenol in an alkaline broth medium.

II. Colon Bacilli That Have the Faculty of Decarboxylating Histidine, Do Not Catabolize Tyrosine to Phenol.

In the course of our studies, which have extended, now, over several years, we have kept alive five microorganisms, all colon bacilli, that have the faculty of decarboxylating histidine to histamine in a medium in which an acid reaction is developed. These microorganisms have previously been shown not to catab-

olize tyrosine in a synthetic salt medium. Section II of Table I is a summary of the results obtained when these organisms are grown in an ascitic fluid broth medium to which tyrosine has been added. A survey of the table shows the following.

1. These organisms do not have the faculty of catabolizing tyrosine to phenol. In this respect they can be sharply differentiated from the colon bacilli whose catabolic activities were summarized in Section I.

2. Aromatic hydroxy acids and tyramine were not produced in any case.

We can conclude, therefore, that colon bacilli that have the faculty of converting histidine into histamine do not catabolize tyrosine. The same enzymatic activity that enables the organism to decarboxylate tyrosine to tyramine will, under suitable conditions, result in the production of phenol from tyrosine. In Section IV of this paper we shall endeavor to show what these conditions are.

III. Colon Bacilli That Do Not Decarboxylate Either Histidine or Tyrosine, Do Not Catabolize Tyrosine to Phenol.

The bacilli used were isolated from human feces about 15 months ago. At that time, none of these five organisms had the faculty of decarboxylating either histidine or tyrosine. The results obtained when these "neutral" coli were grown on an ascitic fluid broth medium are summarized in Section III of Table I.

A survey of the table shows that whereas four of these organisms did not produce phenol, which is what we expected, one of them, Coli CS. 4 converted 34 per cent of the tyrosine into phenol.

We started this series of experiments with the conviction that any microorganism that had a faculty for decarboxylating tyrosine would produce phenol in a broth medium and that the organisms that had a faculty for decarboxylating histidine would not catabolize tyrosine. Just what the "neutral" coli would do was problematic but we thought that some of these, even though they had no decarboxylating faculty, might, nevertheless, convert tyrosine into phenol in a broth medium. At first sight our one anomalous result seemed to be a realization of this possibility; but subsequent experiments have shown that CS.4 has *acquired* the faculty of decarboxylating tyrosine so that it now converts 80

TABLE II.
Showing That Tyrosine Is Catabolized to Tyramine in Acid Solution and to Phenol in an Alkaline Solution.

Experiment No.	Composition of the media.	Tyrosine converted into tyramine. per cent	Tyrosine converted into phenol. per cent	Unchanged tyrosine. per cent	0.1 N HCl neutralized by NH ₄ OH from the entire test solution.	Reaction of the medium.		Remarks.
						Before incubation.	After incubation.	
						pH	pH	
1	Salt medium S.*. 200 cc. Glycerol..... 1 "	91	None.	10	32.5	8.0	5.2	91 per cent of tyrosine converted into tyramine. Phenol absent. pH was reduced to 5.2.
2	Salt medium S... 200 cc. Glycerol..... 1 " Na ₂ HPO ₄ 4 gm.	None.	30	None.	20.0	8.0	7.4	Tyramine absent. 30 per cent of tyrosine converted into phenol. Reaction of the medium was alkaline throughout.
3	Salt medium S... 200 cc.	None.	64	None.	33.0	8.0	8.0	Tyramine absent. 64 per cent of tyrosine converted into phenol. Reaction of the medium was alkaline throughout.
4	Salt medium S... 200 cc. Leucine..... 0.2000 gm.	None.	26	None.	34.4	8.0	8.0	Tyramine absent. 26 per cent of tyrosine converted into phenol. Reaction of the medium was alkaline throughout.

5	Same as in Experiment 3, but containing no NH_4Cl or KNO_3 .	None.	65.5	None.	6.3	8.0	8.0	Tyramine absent. 65.5 per cent of tyrosine converted into phenol. Reaction of medium was alkaline throughout.
6	Peptone broth... 190 cc. Ascitic fluid..... 10 " Tyrosine..... 0.2000 gm.	None.	87	None.	72.5	7.6	7.6	Tyramine absent. 87 per cent of tyrosine converted into phenol. Reaction of the medium was alkaline throughout.
7	Same as No. 6; but contained in addition, 1 cc. of glycerol.	100	None.	None.	20.0	7.6	5.0	Tyrosine converted quantitatively into tyramine. Phenol absent. pH was reduced to 5.0.

* Salt medium S contains:

	gm.
Tyrosine.....	0.2000
NH_4Cl	0.1000
KNO_3	0.1000
KH_2PO_4	0.4000
NaCl	0.8000
Na_2SO_4	0.0200
NaHCO_3	0.4000
CaCl_2	0.0100

Made up with water to a total aqueous volume of 200 cc.

per cent of tyrosine into tyramine when grown in a synthetic salt medium. This organism, therefore, properly belongs in Group I and represents an additional substantiation of the conclusions drawn about these organisms.

Our results indicate that the "neutral" coli are as incapable of catabolizing tyrosine to phenol as are the specific histamine producers; but the number of experiments is too small to warrant our making a statement that would apply to this entire group of bacilli.

IV. Phenol Is Produced Only in an Alkaline Medium, Tyramine Is Formed Only in an Acid Medium.

From our previous experiments the impression was gained that the production of phenol from tyrosine was associated with an alkaline reaction of the medium and that the production of tyramine was associated with an acid reaction of the medium. Table II is a summary of a series of experiments that strongly indicate that the above deductions are correct.

In a salt medium (Experiment 1) to which glycerol had been added and in which an acid reaction developed, *91 per cent of the tyrosine was converted into tyramine*; the unconverted tyrosine was recovered unchanged. When this identical medium was augmented by the addition of Na_2HPO_4 , which left the nutrient conditions unchanged (except for the additional PO_4 and a slight increase in the alkalinity of the medium) but which prevented the development of an acid reaction, *not a trace of tyramine was produced and now 30 per cent of the tyrosine was converted into phenol and 70 per cent of tyrosine disappeared without leaving a clue as to its catabolism*. The medium was colored *brown* in this case, which suggested the presence of phenol oxidation products.

These experiments show that glycerol is not, in itself, conducive to tyramine production. It is the acidity developed from the glycerol that induces the decarboxylation. Conversely, phenol production does not depend upon an absence of available carbon but seems to depend upon the alkalinity of the medium.

Experiments 3, 4, and 5 are further corroborations of this latter statement. In No. 3, tyrosine was the only available source of carbon. The microorganisms multiplied very meagerly in this

medium, but converted 64 per cent of the tyrosine into phenol; 36 per cent of tyrosine disappeared. The reaction was alkaline.

Identical results were obtained when an available source of nitrogen, other than tyrosine, was *not* present. Again the medium had an alkaline reaction (Experiment 5).

When the organisms were grown in an ascitic fluid broth medium 87 per cent of the tyrosine added was converted into phenol (Experiment 6). The reaction was alkaline.

When glycerol was added to a second portion of this same broth medium, sufficient acid was produced from the glycerol, by the microorganisms, to reduce the pH from 7.6 to 5.0 (Experiment 7). *In this case not a trace of phenol was produced; but the tyrosine was converted quantitatively into tyramine.*

CONCLUSIONS.

The experiments described in Parts I to III illustrate, anew, the specificity of the enzymatic activity of microorganisms. The one group of colon bacilli has a specific faculty for catabolizing tyrosine so that tyramine is produced in an acid medium and phenol is produced in an alkaline medium. This group of organisms does not have the faculty of decarboxylating histidine. The second group of colon bacilli has a specific faculty for catabolizing histidine. In an acid medium, histamine is produced. In an alkaline medium histidine is either not catabolized, or products are formed that no longer contain a typical imidazole ring. These organisms do *not* catabolize tyrosine. The third group of colon bacilli does not have the faculty of decarboxylating either of these amino acids.

The specificity, illustrated in the above experiments as holding good for two amino acids, may not be an *absolute* specificity. It is possible that the amino acids can be divided into two or possibly three groups with respect to this enzymatic activity. Certain of the amino acids may be catabolized by the group of organisms that catabolize tyrosine, whereas other amino acids may be catabolized by the organisms that act specifically on histidine. Some of the amino acids may be neutral, in this respect, and may undergo no change with either class of microorganisms.

Experiments along this line, which are now in progress, may lead us to a clearer concept of the interatomic forces that are operative

in the amino acid molecule, for it seems almost inevitable that the specific action of the two groups of microorganisms studied above, for two amino acids that are so similar in composition, must be due to an interatomic difference in the forces that bind the atoms of the side chain. Any of the catabolic products obtained, histamine, tyramine, or phenol, must be due to an action on the side chain of the molecule. These side chains are structurally identical, hence the difference must be interatomic.

The experiments described in Part IV show that with identical nutrient conditions, tyramine is produced in a medium that becomes acid in reaction and phenol is produced in a medium that remains alkaline. *The determining factor seems to be the reaction of the medium rather than the nutrient conditions.* Thus, phenol is produced in an alkaline ascites broth medium, in a simple salt medium containing tyrosine but no glycerol, and in a more complex synthetic medium containing salts, tyrosine, glycerol, and sufficient Na_2HPO_4 to maintain an alkaline reaction. When the Na_2HPO_4 is left out of the last medium, an acid reaction develops rapidly and, in this case, *tyramine* is produced, but *phenol is not produced.*

In a medium having a solid or semisolid consistency, such as we find in the large intestine of man, it is possible for areas of acidity and of alkalinity to develop in fairly close proximity due to a difference in chemical composition. Under such conditions, phenols and amines might be produced simultaneously and this seems to be the case. That the normal intestinal contents of man contain phenol is a generally recognized fact. In our next communication we shall show that *histamine is also a normal constituent of human feces.*

SUMMARY.

1. Colon bacilli can be divided into three classes with respect to their decarboxylase activity; namely, (1) those that decarboxylate tyrosine to tyramine, (2) those that decarboxylate histidine to histamine, and (3) those that do not have the faculty of decarboxylating either of these amino acids. A strain that decarboxylates tyrosine will not decarboxylate histidine and *vice versa*.

2. There is no relationship between decarboxylase activity and sugar fermentation. The customary bacteriological classification gives no clue as to the decarboxylase activity of the microorganisms.

3. The decarboxylase activity is a protective mechanism and is resorted to, by the microorganism, when the accumulation of H ions within the bacterial protoplasm is incompatible with its normal life processes. Histamine and tyramine are, therefore, produced in any medium in which a strong acid reaction is developed, regardless of the composition of the medium (providing, of course, that the appropriate amino acids and the correct organisms are present).

4. In a broadly buffered medium, or one in which the development of acidity is impossible, tyrosine is catabolized to phenol; but only by those bacilli that have the faculty of decarboxylating tyrosine to tyramine in an acid medium. Other colon bacilli do *not* catabolize tyrosine in alkaline solution.

5. The faculty for decarboxylation, when once possessed by the microorganisms of the colon group, seems to be retained indefinitely. The faculty of decarboxylating tyrosine can be acquired by certain of these microorganisms, when they are grown, for some time, on a glycerol agar medium.

STUDIES ON PROTEINOGENOUS AMINES.

XX. ON THE PRESENCE OF HISTAMINE IN THE MAMMALIAN ORGANISM.

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INTRODUCTION.

The work reported in Papers XVII and XVIII of this series^{1, 2} brings definite proof that the normal bacterial inhabitants of the alimentary tract have the faculty of converting the innocuous amino acids, histidine and tyrosine, into the highly toxic amines, histamine and tyramine. Our previous work^{2, 3, 4} has shown that this conversion is always associated with a medium in which an acid reaction is developed. The feces and cecal content, because of their solid or semisolid consistency, may contain areas of acidity and alkalinity; and one would expect that amines must, necessarily, be a normal constituent of the content of at least the large intestine. Meakins and Harington⁵ were able to demonstrate the presence of a substance that was pharmacologically identical with and chemically very similar, if not identical, to histamine in the contents of the ileum and cecum; but they were unable to demonstrate the presence of such compounds in the feces.

In 1920⁶ we published a purely chemical method for estimating histamine in protein and protein-containing matter. We

¹ Koessler, K. K., and Hanke, M. T., *J. Biol. Chem.*, 1924, lix, 835

² Hanke, M. T., and Koessler, K. K., *J. Biol. Chem.*, 1924, lix, 855.

³ Koessler, K. K., and Hanke, M. T., *J. Biol. Chem.*, 1919, xxxix, 539.

⁴ Hanke, M. T., and Koessler, K. K., *J. Biol. Chem.*, 1924, lix, 867.

⁵ Meakins, J., and Harington, C. R., *J. Pharmacol. and Exp. Therap.*, 1921, xviii, 455.

⁶ Hanke, M. T., and Koessler, K. K., *J. Biol. Chem.*, 1920, xliii, 543.

began, shortly afterward, to examine cecal and fecal contents for histamine and had completed several of our analyses when the paper by Meakins and Harington appeared.

Their paper illustrates what we have also found to be true; namely, histamine is easily and firmly adsorbed by finely divided solids. Their mode of procedure consisted in treating the material to be examined with HgCl_2 in acid solution which left most of the solid matter undissolved. The mixture was filtered and the residue discarded. Now it is just this residue, as we shall show, that contains most if not all of the histamine; hence it is not surprising that they were unable to demonstrate the presence of histamine in feces. The cecal and ileal content contains far less solid matter; hence a small portion of the histamine present remains in the aqueous extract, where they found it.

Meakins and Harington were seduced into the use of basic lead acetate because of its excellent clarifying qualities. We also tried this method, but abandoned it because the losses are enormous. Histamine is adsorbed to a considerable extent by copious precipitates of lead salts.

Our first series of experiments showed that the method published in 1920 did not give quantitative yields when applied to feces. The recovery was about 60 per cent (see Experiment 1). We tried to reduce the losses in many ways, but have finally returned, with a few minor modifications, to the original method because it gives the most trustworthy results.

The first step in our original method⁶ consisted of a digestion of the material with 75 per cent alcohol, which had been slightly acidified with acetic acid, thus dividing the material into two fractions, an alcoholic extract and an alcohol-insoluble residue. This step was introduced because we hoped that the free histamine would pass into the alcohol leaving behind only the peptamine histamine. This process has been abandoned because, in the presence of a large amount of insoluble matter, the uncombined histamine, *even when added as hydrochloride*, is very largely adsorbed by the insoluble residue and only fragments thereof appear in the alcoholic extract. In our earlier experiments, this separation was carried out. The results are given in the text.

The final step in the original process⁶ consisted in a methyl alcohol-chloroform purification which was found to remove the

substances that interfere with the colorimetric determination of histamine. This purification process cannot be applied in the case of feces because most of the histamine is carried down by the chloroform-insoluble residue.

The remaining eight steps of our original process have been found to give satisfactory results.

Mode of Procedure.⁷

The material to be examined for histamine is treated with 10 to 20 parts, by weight, of 37 per cent HCl and hydrolyzed by boiling for 30 hours over an electrically heated sand bath.

The hydrolysis mixture always contains a large amount of black, insoluble residue which undoubtedly adsorbs considerable histamine. This solid can be removed, by filtration, at this time or it can be removed later along with the humin fraction. The final yields of histamine are identical irrespective of whether this solid is removed from an acid or from an alkaline medium. We have usually removed the solid as a part of the humin fraction.

The hydrochloric acid is removed by distillation *in vacuo* at 60°, from the same flask.

The residue is dissolved in 10 to 20 parts, by weight, of water and the mixture treated with an excess of lime and a volume of alcohol equal to one-half the volume of the water added. The mixture is then subjected to a distillation *in vacuo* which removes ammonia and is finally filtered, to remove the humin.

The alkaline filtrates so obtained are highly colored; but any attempt to remove the color at this time leads invariably to a decided loss of histamine. The coloring matter is progressively lost in the subsequent steps of the process and in those few cases where it persists to the end, it can be easily removed, with but little loss of histamine, by precipitating mercuric sulfide in the solution. The details are given later.

The alkaline filtrate is acidified with HCl. The change in reaction from alkaline to acid is usually associated with a sharp reduction in color, the tint changing from red-brown to yellow-brown. This liquid is concentrated, on the water bath, and treated with phosphotungstic acid in acid solution.

⁷ A detailed account of this process is given in Paper VIII of this series.⁶

The phosphotungstates are removed by filtration. The carefully washed precipitate, which contains the histamine, is transferred, in small portions, to a large mortar and thoroughly macerated with a hot, concentrated solution of barium hydroxide. In this way the baryta is brought into intimate contact with the insoluble phosphotungstates and the possibility of an incomplete conversion is avoided. The mixture finally obtained is digested for 1 hour on the water bath, filtered, and the barium removed from the filtrate with sulfuric acid.

The resulting acid filtrate is concentrated on the water bath. The residue is diluted with water, alkalinized, and extracted exhaustively with amyl alcohol, which removes histamine quantitatively.

The combined amyl alcohol extracts are extracted five times with $N H_2SO_4$. Histamine passes quantitatively into the aqueous acid layer. This is neutralized with barium hydroxide, filtered, and concentrated on the water bath in a glass dish. The residue is dissolved in water, the solution alkalinized and extracted with amyl alcohol as above. The amyl alcohol extraction is one of the most important steps in our process and should be repeated twice (three complete extractions in all). Since histamine is, ultimately, to be determined colorimetrically, and since histidine gives a color that is easily confused with that of histamine, it is essential that every trace of the amino acid be removed.⁸ The final amyl alcohol extract does not contain histidine. Coloring matter and substances that interfere with the colorimetric estimation of histamine are very largely removed in this way. Histamine is never lost when these extractions are properly conducted.

The residue finally obtained is dissolved in 50 to 100 cc. of water. The solution is used for colorimetric determinations as volumes of the above test liquid with definite proportions of 1.1 previously described.⁹ This consists briefly in mixing known

* If this separation is omitted, a positive Pauly reaction or a positive Knoop reaction cannot properly be interpreted as indicating the presence of histamine. Histidine not only gives these tests but the usual phosphotungstic acid and silver fractionations do not separate histidine from histamine.

* Koessler, K. K., and Hanke, M. T., *J. Biol. Chem*, 1919, xxxix, 597.

per cent Na_2CO_3 and *p*-phenyldiazonium sulfonate and comparing the color so produced with a standard solution containing Congo red and methyl orange.

The colorimetric determinations on the stool extracts obtained by the method just described almost invariably revealed the presence of substances that interfered with the color production. There are two kinds of interfering substances; namely, those that partially or completely prevent the coupling of histamine with *p*-phenyldiazonium sulfonate and those that give greenish colors with the reagent. When the first of these is present, the speed of development of the color is greatly retarded and the color is never maximal. Doubling the volume of test liquid does not double the amount of color obtained. When histamine is added to the test liquid the total amount of color that should be obtained, theoretically, is not developed. The second type of interfering substance is unmistakable because the greenish color changes the normal tint so completely that a comparison with the standard indicator solution is almost impossible.

The presence of either type of interfering substance, and they were almost invariably present in our mixtures, necessitates a silver precipitation (which is step 9 of our original process⁶). The test liquid is treated with an excess of silver nitrate and then with baryta. The insoluble residue is removed by filtration, the precipitate carefully washed with a cold saturated solution of baryta, suspended in dilute HCl, triturated in a mortar, and the resulting mixture digested for 1 hour on the boiling water bath, after adding sufficient of a 20 per cent solution of Na_2SO_4 to remove the barium completely. AgCl and BaSO_4 are removed by filtration. The filtrate is neutralized with sodium hydroxide and concentrated on the water bath. The residue is dissolved in water and reextracted with amyl alcohol as previously described.

In this way a test liquid is finally obtained that is usually colorless and that is also usually free from interfering substances. The final test liquid may, occasionally, be highly colored. In such cases the coloring matter can be removed, with but little loss of histamine, by adding 1.0 gm. of HgCl_2 and saturating the liquid with H_2S . The HgS adsorbs the coloring matter; but does not adsorb appreciable quantities of histamine.

Substances that interfered with the colorimetric determination of histamine were present in two of our final test liquids (Experiments 4 and 5). We tried to remove them in a number of ways; but were unsuccessful.

Analysis of Human Feces for Histamine.

Experiment 1.—H. L. H. This experiment was conducted to ascertain the approximate losses of histamine to which our method is subject. The feces collected, 530 gm., were treated with 1,500 cc. of 20 per cent HCl and hydrolyzed by boiling for 30 hours over an electrically heated sand bath. The acid was removed by distillation *in vacuo*. The residue, so obtained, was dissolved in water. The mixture was diluted, with water, to a volume of 1,000 cc. This liquid was divided into two equal parts. Histamine dichloride, 10 mg., was added to one-half. Both portions were then subjected to the analytical procedure described above.

Untreated Portion.—The total volume of the final test liquid was 50 cc. of which 0.10 cc. gave a color equivalent to 2.5 mm. of the standard (CR-MO) indicator solution and 0.20 cc. gave a color equivalent to 5.0 mm. (CR-MO). The color was exactly like that obtained with histamine. Interfering substances were absent. This color value is equivalent to 0.0017 gm. of histamine dichloride for the entire 50 cc. portion of the test liquid or 0.0034 gm. for the entire 530 gm. of feces.

The Fraction to Which 10 Mg. of Histamine Had Been Added.—The total volume of the final test liquid was 50 cc. of which 0.10 cc. gave a color equivalent to 11.0 mm. (CR-MO) and 0.20 cc. gave a color equivalent to 22.0 mm. (CR-MO). The color was exactly like that obtained with histamine. Interfering substances were absent.

Of the 11.0 mm. of color obtained with the 0.10 cc. portion, 2.5 mm. can be assumed to have been due to the histamine originally present in the feces.¹⁰ The color value due to the added histamine must, therefore, have been equal to 8.5 mm. (CR-MO) for 0.10 cc. of the test liquid. This is equivalent to 0.0057 gm. of histamine dichloride, 57 per cent of the amount originally added. We can safely assume that the histamine determined in the untreated half portion of this same feces was certainly not over 60 per cent of the amount actually present which shows that the 530 gm. of feces must have contained 5.65 mg. of histamine.

We have not corrected our other figures for possible losses; but it is well to keep the fact in mind that our figures are minimal rather than maximal.

¹⁰ This may not be *exactly* correct because the percentage of the total histamine adsorbed may vary with the concentration of histamine present; but an experiment, that we shall discuss later, indicates that a certain *percentage* of the histamine present is adsorbed even when the concentrations of histamine are widely divergent.

Experiment 2.—A second sample of feces from the same person, 560 gm., was subjected to the alcohol separation process. The alcohol-insoluble residue was found to contain 7.5 mg. of histamine and the alcoholic extract contained 10 mg. of histamine, uncorrected for possible losses.

Experiment 3.—Feces of M. H. Moist weight 648 gm. This sample was subjected to the alcohol separation process.

Alcohol-Insoluble Residue.—The nearly colorless residue finally obtained was dissolved in water and diluted to 50 cc., of which 0.05 cc. had a color value equivalent to 13.7 mm. (CR-MO) and 0.10 cc. had a color value equivalent to 27.4 mm. (CR-MO). The color was exactly like that obtained with histamine. Interfering substances were absent. This is equivalent to 0.0183 gm. of histamine dichloride for the entire test liquid.

The Alcoholic Extract.—The residue finally obtained was dissolved in water and diluted to 50 cc. Of this liquid, 0.10 cc. had a color value equivalent to 3.0 mm. (CR-MO) and 0.30 cc. had a color value equivalent to 9.0 mm. (CR-MO). The color was exactly like that obtained with histamine. Interfering substances were absent. This is equivalent to 2.0 mg. of histamine dichloride for the entire test liquid.

The presence of 20.3 mg. of histamine was demonstrated in this sample of feces. The person who furnished the material was in perfect health.

Experiments 4 and 5.—The feces of K. K., 634 gm., and of C. C., 287 gm., were subjected to the identical process used in the first three experiments; but in these cases both types of interfering substances were present in the final test liquid. We were, therefore, unable to determine the amount of histamine present. That histamine was present was indicated by a momentary flare of color that was obtained when the test liquid was allowed to flow into the alkaline reagent. The greenish yellow color that appeared within 1 minute after the test solution had been mixed with the alkaline reagent, completely obliterated the color due to histamine.

Determination of Histamine in Human Cecal Contents.

The cecal contents were drained, from an ileocecal fistula, into a glass bottle to which a measured volume of alcohol had been added. The alcohol concentration was never less than 50 per cent. The mixture was yellow in color and had a sour but not a fecal odor.

Experiment 6.—Mrs. R. A total of 1,200 cc. of cecal matter was collected. The mixture was evaporated to remove alcohol and water. The residue was suspended in 1,500 cc. of 20 per cent HCl and hydrolyzed as usual. The mixture was then analyzed for histamine.

The dark brown residue finally obtained was dissolved in water and diluted to 50 cc. Of this liquid, 0.10 cc. had a color value equivalent to 11.2 mm. (CR-MO) and 0.20 cc. had a color value equivalent to 22.5 mm. (CR-MO). The color tint was masked, somewhat, by the inherent color of

the test liquid. The test liquid was subjected to the $\text{HgCl}_2\text{-H}_2\text{S}$ treatment which gave, finally, a nearly colorless liquid that was again diluted to 50 cc. and used for the colorimetric determinations.

0.10 cc. had a color value of 10.2 mm. (CR-MO).

0.20 " " " " " " 20.4 " (CR-MO).

The color was exactly like that obtained with histamine. Interfering substances were absent. This is equivalent to 0.0068 gm. of histamine dichloride for the entire 1,200 cc. of cecal matter.

Experiment 7.—Mr. M. A total of 600 cc. of cecal matter was collected. This was treated as described under case 6. The pale yellow residue finally obtained was dissolved in water and diluted to 50 cc. of which

0.10 cc. had a color value equivalent to 3.0 mm. (CR-MO).

0.20 " " " " " " 6.0 mm. (CR-MO).

This is equivalent to 0.002 gm. of histamine dichloride for the entire 600 cc. of cecal matter.

Analysis of Human Liver for Histamine.

The liver, weighing 1,550 gm., was obtained from a man who had died suddenly in an accident. The liver was removed 4 hours after death. It was ground up in a meat chopper, treated with 2,000 cc. of 37 per cent HCl , and hydrolyzed. HCl , NH_3 , and humin were removed in the manner previously described. The liquid finally obtained was diluted to 2,000 cc. The analysis was conducted on 500 cc. of this liquid.

The colorless residue finally obtained was dissolved in water and diluted to 50 cc. *Not a trace of color was obtained with 1.00 cc. of this test liquid.* Interfering substances were absent.

This normal human liver did not contain histamine.

Estimation of Histamine in the Feces, Portal Blood, and Liver of a Dog.

The dog, a Scotch collie weighing 35 kilos, was fed meat for 5 days. On the last day, 150 gm. of feces were collected. The dog was anesthetized and prepared so that blood could be obtained from the portal vein. 300 cc. of blood were collected. The animal was completely exsanguinated. The liver, weighing 475 gm., was removed, ground up in a meat chopper, and treated with 1,500 cc. of alcohol.

The Histamine Content of Dog Feces.—The feces, 150 gm., were extracted repeatedly with 75 per cent alcohol. Each of the

two fractions so obtained was analyzed for histamine. *The alcohol-insoluble residue was found to contain 0.3 mg. of histamine. The alcoholic extract yielded 5.0 mg. of histamine.*

Pharmacological experiments also indicated the presence of approximately these amounts of histamine. This is the only case we have had in which the histamine passed almost quantitatively into the alcoholic extract.

Examination of Portal Blood for Histamine.—The blood, 300 cc., was mixed with 900 cc. of 95 per cent alcohol. The mixture was filtered. Each of the two fractions so obtained was analyzed for histamine.

The colorless test liquids finally obtained with each fraction gave no color with an alkaline solution of *p*-phenyldiazonium sulfonate. The liquids were pharmacologically inactive.

This sample of portal blood did not contain histamine in recognizable amounts.

The Histamine Content of Dog Liver.—The liver, 475 gm., was ground up in a meat chopper and treated with 1,500 cc. of alcohol. The mixture was filtered. Each of the two fractions so obtained was analyzed for histamine. *The alcohol-insoluble residue yielded 6 mg. of histamine. The alcoholic extract yielded 1.25 mg. of histamine.*

Examination of the Intestinal Contents, Intestinal Tract, and Liver of the Normal Guinea Pig for Histamine.

Two normal, well fed guinea pigs, weighing approximately 800 gm. each, were asphyxiated. The liver and the entire alimentary tract, from the esophagus to the anus, were removed. The contents of the stomach and intestines were carefully washed out with distilled water. The three fractions so obtained, intestinal tract, intestinal contents, and liver, were separately hydrolyzed with 20 per cent HCl and analyzed for histamine.

Histamine was not found in any of the fractions.

The intestinal contents, the intestinal tract, and the liver of the normal guinea pig do not contain histamine in recognizable amounts.

CONCLUSIONS.

Histamine appears to be a normal constituent of the contents of the large intestine of man and dog. The fact that histamine

was found in the liver of a dog suggests that it must have been transported there from the intestines by way of the portal blood stream. Our failure to find histamine in the portal blood is of little significance because the blood flow is so rapid that only traces of histamine could be present at any time. The dog's liver seems to store histamine. The negative result obtained with the human liver merely shows that this organ does not invariably contain histamine. It will be necessary to repeat this experiment a number of times before any definite conclusion can be reached. Histamine seems not to be a normal constituent of the herbivorous guinea pig. This is rather to be expected because of the alkaline character of the diet and its low histidine content. A detailed discussion of the significance of these results, and of those obtained previously, will be found at the end of our next paper, No. XXI of this series.

SUMMARY.

1. A purely chemical method is described for the estimation of histamine in the liver, intestinal contents, and feces of animals.
2. Human feces, 500 to 600 gm., from normal individuals, have been found to yield from 6 to 20 mg. of histamine. Cecal content, 600 and 1,200 cc., contained 2 and 7 mg. of histamine, respectively. The one human liver examined did not yield histamine.
3. Dog feces, 150 gm., contained 5.3 mg. of histamine. The liver of this same dog yielded 6 mg. of histamine.
4. The intestinal contents, intestinal tract, and liver of two normal guinea pigs did not yield histamine.

STUDIES ON PROTEINOGENOUS AMINES.

XXI. THE INTESTINAL ABSORPTION AND DETOXICATION OF HISTAMINE IN THE MAMMALIAN ORGANISM.

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Work on the proteinogenous amines thus far reported shows that the human intestinal tract is normally inhabited by microorganisms which have the faculty of decarboxylating histidine to histamine and tyrosine to tyramine. We were able to show that this amine production (decarboxylase activity) is a specific function of certain species of bacteria and of certain strains within the species; microorganisms which decarboxylate histidine do not decarboxylate tyramine and *vice versa*. The decarboxylation of the amino acids, histidine and tyrosine, and probably of other amino acids resulting in amine production, is thus a normal pathway in the catabolism of amino acids in the intestinal tract which occurs side by side with deamination. Histamine (and probably also tyramine) is a normal constituent of the cecal and fecal matter of man; *i.e.*, of the large intestines.

This last point, the actual demonstration that relatively large amounts of histamine are present in the intestinal tract of healthy men, invited further investigation into the fate of this amine in the organism. For it deserves the meditation of the physiologist as well as of the clinician, how it is possible that such quantities of a highly poisonous substance could be harbored within the organism without producing symptoms of intoxication. There are several explanations which present themselves.

1. The histamine is detoxicated within the intestinal lumen. The contents of the intestine, by means of their bacterial and cellular enzymes, might produce rapid changes in the structure and constitution of the amine of such nature that it is deprived of

its toxicity. On this assumption the histamine found by us in the intestinal contents would represent only a transitional stage in protein metabolism and would not be an end-product.

2. Histamine is not absorbed. It might, for example, be so firmly adsorbed by the solid constituents of the intestinal contents that it is not free to pass through the intestinal wall.

3. Histamine is absorbed, but during absorption is at once detoxicated by the animal tissues (*e.g.* by the liver or by the cells of the intestinal wall itself).

Certain phases of this problem of the intermediary metabolism, fate, mode of absorption, and detoxication of imidazole ethylamine, have been approached by previous investigators and before we report our own attempts directed toward solution of these questions it might be well to review briefly those papers which have a direct bearing on our subject.

Dale and Laidlaw,¹ investigating the fate of histamine by perfusion experiments through the liver, obtained some evidence of its disappearance, but the limit of the destructive power of the liver appeared to be reached very quickly. Neither by adding one large dose (200 mg. or more) nor by successive small additions could they get evidence of a total destruction amounting to more than 10 mg.

In a paper on the mode of action of histamine on intravenous injection Oehme² showed that many multiples of the acute lethal dose of a dilute histamine solution might be injected provided the injection is done slowly. He showed that a very rapid detoxication must be taking place in the animal organism and demonstrated further that the difference between peripheral *venous* injection and that through the portal way disappears completely on slow injection. All the previously conducted comparison experiments have, as far as we know, been carried out in this way; *i.e.*, a peripheral venous injection has been compared with a portal injection. Dr. H. G. Wells has called our attention to the fact that these two routes are hardly comparable because in one case only one capillary system is traversed by the poison; namely, the pulmonary; in the other instance,

¹ Dale, H. H., and Laidlaw, P. P., *J. Physiol.*, 1919, xli, 318.

² Oehme, C., *Arch. exp. Path. u. Pharmacol.*, 1913, lxxii, 76.

however, two are traversed, the hepatic and pulmonary. It should be more correct to compare the injection into the femoral *artery* with one in the portal system for in this way two capillary systems have to be traversed in both instances. We have adopted this mode of procedure in our work.

A very interesting and important piece of work was reported by Guggenheim and Löffler in 1916.³ These investigators showed—extending the previous work of Ewins and Laidlaw on *p*-hydroxyphenylethylamine—that most proteinogenous amines, such as isoamylamine, phenylethylamine, *p*-hydroxyphenylethylamine, indolethylamine, and imidazole ethylamine, are detoxicated in the animal organism. The detoxication proceeds through deamination and oxidation by way of the alcohol to the aldehyde and finally results in the formation of fatty acids of the same number of C atoms as the amines. Only the catabolism of histamine (β -imidazole ethylamine) to imidazolacetic acid could not be proved. If 1 gm. of histamine in solution was perfused for several hours through a rabbit liver, no considerable destruction of the amine could be demonstrated by biological methods.

The most recent work on this subject is reported by Meakins and Harington.⁴ These investigators, studying the absorption of histamine from the intestine in the *cat*, found that histamine introduced as hydrochloride, was absorbed from all parts of the intestinal tract. The rate of absorption was most rapid from the ileum, somewhat less from the duodenum, and very much less, but still quite definite, from the cecum and stomach. Regarding the part which the liver played in the destruction of histamine after absorption these authors, on the basis of the work with animals in which Eck fistulas were made, feel that the liver exercises a protective function, which is ascribed to the cushioning effect of its extensive capillary network, which prevents a sudden flood of the substance from reaching the general blood stream and thus is regarded as more mechanical than chemical.

Our own experiments directed toward the solution of these problems were of two kinds:

³ Guggenheim, M., and Loeffler, W., *Biochem. Z.*, 1915-16, lxxii, 325.

⁴ Meakins, J., and Harington, C. B., *J. Pharmacol. and Exp. Therap.*, 1921, xviii, 455; 1923, xx, 45.

1. Dogs and guinea pigs were fed known amounts of histamine dichloride. After the animals had been kept under observation for some time, they were killed. The intestinal contents, the intestinal tract, and the liver were then examined for histamine. In this way we were able to ascertain the approximate rate at which the amine disappeared from the intestinal tract.

2. Large dogs were anesthetized and prepared so that blood pressure and respiratory tracings could be obtained. Dilute solutions of histamine dichloride were then injected, at definite rates, into the saphenous vein, the femoral artery, the mesenteric vein, the splenic vein, and the duodenum. It was possible for us, in this way, to determine the minimum effective dose of histamine for the venous system, the buffer effect of the capillary network of the dog's leg, and the effectiveness of the liver as a detoxicating organ.

I. On the Absorption and Disappearance of Histamine from the Alimentary Tract.

Mode of Procedure.

A gelatin capsule containing the finely powdered histamine dichloride was placed in the posterior pharynx of guinea pigs in such a manner that it passed into the esophagus intact. The animals were carefully watched to make certain that they had swallowed the capsule. Histamine has thus to be introduced directly into the stomach, for comparatively small doses will cause death when the amine comes in contact with the mucous membrane of the mouth, through which it is rapidly absorbed. In another set of experiments a watery solution of histamine dichloride was introduced directly into the stomach of young dogs by means of a stomach tube.

The analytical procedure used in determining histamine was that described in the preceding paper.

Experiment 1.—Approximately 80 per cent of the histamine present in the intestinal contents of a guinea pig can be recovered by our method.

Two large guinea pigs, weighing each approximately 800 gm., which had been fed exclusively on carrots, were asphyxiated. The entire alimentary tract was removed and the contents of the stomach and intestines washed out with 0.85 per cent salt solution.

To the contents of the alimentary tract of one animal were added 100 mg. of histamine dichloride. The contents from the other guinea pig were treated with 20 mg. of histamine dichloride. The two mixtures were then separately hydrolyzed and analyzed for histamine.

Histamine added	Histamine recovered.	Efficiency of method.
<i>mg.</i>	<i>mg.</i>	<i>per cent</i>
100	80	80
20	16.8	84.3

The methods seems, therefore, to recover approximately 80 per cent of the histamine present irrespective of the initial concentration.

Experiment 2.—Histamine is not changed by a 24 hour incubation with intestinal contents.

100 mg. of histamine dichloride in solution were intimately mixed with the contents of the stomach and intestines of a guinea pig which weighed about 800 gm. and the mixture was then incubated for 24 hours at 37°C. At the end of this time it was hydrolyzed and analyzed for histamine. Exactly 80 mg. of histamine dichloride were recovered, 80 per cent of the amount originally added.

We proved, in Experiment 1, that our method is 80 per cent efficient when applied to this kind of material; hence we are justified in concluding that the constituents of the intestinal contents do not modify or destroy histamine so that the amine is unrecognizable. The first of our speculative possibilities to account for the disappearance of histamine seems thus to be eliminated by this experiment.

Experiment 3.—100 mg. of histamine, when fed to a guinea pig, does not cause death. The amine disappears from the alimentary tract within 24 hours.

Histamine dichloride, 100 mg. enclosed in a gelatin capsule, was fed to an 800 gm. guinea pig. During the 1st hour the animal showed some signs of discomfort. It seemed depressed, sneezed occasionally, scratched its nose, and salivated profusely. These symptoms had disappeared completely at the end of 2 hours. The animal's normal appetite was reestablished. The following morning, 24 hours after the histamine had been ingested, the guinea pig seemed quite normal. It was killed by asphyxiation; the entire alimentary tract was removed, free from adhering fat and omentum, slit open, and the contents carefully removed by washing with physiological salt solution. The alimentary tract and the contents were separately hydrolyzed and analyzed for histamine. *The alimentary tract did not contain histamine. The contents contained only 1.8 mg. In some way, 98.4 mg. of histamine disappeared from the alimentary tract without causing death and without eliciting marked symptoms.*

Experiment 4.—100 mg. of histamine, when fed to a guinea pig, have disappeared from the alimentary tract to the extent of 61.8 per cent within 2 hours.

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Experiment 3 was repeated excepting that the animal was killed 2 hours after the histamine had been injected and that the liver was removed as well as the alimentary tract, and analyzed for histamine. This animal neither vomited nor defecated during this time interval. The stomach was found to be acutely dilated with fluid. Solid matter was almost absent.

	Histamine found.	Efficiency of method.	Histamine present.
	<i>mg.</i>	<i>per cent</i>	<i>mg.</i>
Intestinal contents.....	26 8	80	33.5
Alimentary tract.....	4 7	100(?)	4 7
Liver.....	4 5	100(?)	4 5
Total.....			42.7

In this case, 66.5 mg. of histamine dichloride disappeared from the intestinal contents; 4.7 mg. of this were surely in the *tissue* of the alimentary tract and 4.5 mg. had been transported to the liver. The remaining 57.3 mg. were not accounted for. If we assume that the histamine was absorbed, as such, to pass into the portal blood stream or into the lymphatic circulation and thence to the systemic circulation, the body of this guinea pig was being injected with histamine at the rate of 0.5 mg. per minute. It is well known that *one* intravenous injection of 0.5 mg. of histamine dichloride will kill a guinea pig of this size. We are, therefore, forced to conclude that the histamine, as such, could not have been thrown into the general circulation at any such rate of speed.

The experiment indicates that the histamine passed *into* the intestinal wall as histamine, for we found some of it there. It is also clear that *some* of the histamine reached the liver as histamine, because we found it in this organ. This experiment tells us nothing, however, about what happens to the major part of the amine after it leaves the lumen of the intestine. Two possibilities present themselves:

1. The amine may be absorbed and pass into the liver *as such*. In this case the liver must possess a highly developed faculty for depriving histamine of its toxicity, because, if such large doses of amine were to pass into the systemic circulation, death would be the inevitable outcome.

2. Histamine may be detoxicated in its passage through the intestinal wall. In this case we should be ascribing to the thin intestinal wall a function that seems almost prodigious and we recall hesitatingly how other functions that have been ascribed

to this membrane from time to time have all proved untenable (*e.g.* the synthesis of proteins).

But in this instance it is possible that we may have to deal with the phylogenetic development of a protective mechanism, for histamine exerts a very stimulating effect upon the musculature of the intestines eliciting violent contractions. The ability of the intestinal mucosa to detoxicate amines might have had to be acquired in the phylogenesis of man.

Before we turn to a consideration of the above problems, we shall describe a typical example of the conditions prevailing when histamine is fed to a dog, from which it becomes clear that the results obtained with the guinea pig are more general in their application.

Experiment 5.—500 mg. of histamine when fed to a 5 kilo dog have disappeared from the alimentary tract to the extent of 53 per cent within 2 hours.

After convincing ourselves that puppies show no signs of discomfort after the ingestion of 500 mg. of histamine dichloride, we introduced 500 mg., dissolved in 200 cc. of water, by stomach tube into a puppy weighing 5.2 kilos. There was no reaction of any kind. The animal did not vomit. The dog was killed with illuminating gas 2 hours after the amine was ingested.

The entire alimentary tract was removed and freed from fat and omentum. A ligature was placed tightly at the cardiac and pyloric ends of the stomach to prevent loss of material. The intestine was cut off just below the stomach. The material was, in this way, divided into two fractions; namely, the stomach and the intestines. Each of these was separately hydrolyzed and analyzed for histamine.

The stomach and contents weighed 500 gm. This organ was acutely dilated with fluid. Solid matter was practically absent. The contents were not separated from the stomach wall; the analysis was conducted on the entire material.

The stomach and contents contained 147 mg. of histamine dichloride.

Since the material was simple in composition, mostly fluid, this figure is probably not subject to corrections. This is equal to 29.4 per cent of the amount ingested.

The alimentary tract, below and exclusive of the stomach, weighed 520 gm. This was hydrolyzed and analyzed for histamine. The contents were not separated from the tract.

The intestines contained 53.2 mg. of histamine dichloride which is 10.64 per cent of the amount originally ingested.

Since this material was something like human feces in consistency, for which the efficiency of our method has proved to be 60 per cent, the maximum amount of histamine that *could* have been present in the intestine is 88.65 mg., which is 17.73 per cent of the amount originally ingested.

The total recovery was 235.6 mg.; hence 264.4 mg. of histamine dichloride were absorbed in 2 hours. The average absorption rate was, therefore, 2.2 mg. per minute; but we know that, if histamine were injected into the systemic circulation at anything approaching this rate of speed, severe symptoms of intoxication would undoubtedly have developed.

The feeding experiments lead always to the same conclusion. It is unreasonable to assume that the histamine absorbed passes into the general circulation as such. The compound must be removed from the general circulation in some way. We have already indicated, under Experiment 4, that there are only two possible explanations. The amine must be detoxicated either in its passage through the intestinal wall or subsequently by the liver. The other tissues of the body unquestionably have some power of detoxicating this amine; but this in itself would not be sufficient to account for our results.

So much speculation has surrounded the liver as a detoxicating organ that, in spite of the convincing experiments of Meakins and Harington,⁴ we decided to investigate, in a different way, the rôle of the liver as a "detoxicator" of histamine.

*II. On Certain Factors Involved in the Extent of Pharmacodynamic Action of Histamine Following Its Injection into the Circulation.*⁵

The toxic action of histamine on intravenous injection in the common laboratory animals is well known since the extensive studies of Dale and his coworkers. While the pharmacological effect of the poison is very similar in its symptoms in the different species, the minimal lethal dose of histamine on intravenous injection varies considerably in different animals. For guinea pigs and rabbits 0.6 to 0.8 mg. of histamine per kilo constitutes the fatal dose.

Working with dogs we found that 30 mg. of histamine per kilo body weight is insufficient, in unanesthetized animals, to produce death.⁶

We were, however, more interested to establish the minimal effective dose of histamine in the dog; *i.e.*, the smallest quantity

⁵ In the experimental part of this work we were ably assisted by Dr. H. L. Huber.

⁶ We were assisted, in these experiments, by Dr. Julian H. Lewis.

of the amine which on intravenous injection would still produce a definite fall in blood pressure.

Large dogs were used in this work (10 to 20 kilos). In our first experiments ether preceded by an injection of morphine was used as anesthetic. We found, however, that the respiration and blood pressure would remain at a more constant, normal, level when barbital⁷ was used as anesthetic. Even with this anesthetic it is advisable to use a small amount of ether while the intestines are manipulated.

The dog, after anesthetization, was prepared so that blood pressure and respiratory tracings could be made simultaneously on the drum of the same kymograph. The injections were made with a Woodyatt pump.⁸ The apparatus had to be arranged so that we could switch from salt to histamine solution, or *vice versa*, within a few seconds. To accomplish this two pumps were used, one for histamine and one for salt solution. The solutions were fed to the machines from burettes so that the rate of injection could be easily followed. All solutions were warmed to 40°C. before they passed into the animal's circulation. The feed pipes, from the pump to the animal, were composed of glass. Rubber connections, where such were necessary, were as short as possible.

Many animals were used in these experiments, but we will give here only a few graphs which illustrate sufficiently the points we wish to make.

Fig. 1 shows the effect of injecting histamine into the saphenous vein. The minimum effective rate was 0.0027 mg. *per minute* per kilo body weight. When the injection was made at the rate of 0.0051 mg. *per minute* per kilo body weight, there was a pronounced fall in blood pressure, but no respiratory embarrassment. When the injection rate was increased to 0.0108 mg. *per minute*, the animal reacted with a marked fall in blood pressure and considerable respiratory embarrassment. These experiments were repeated a large number of times on dogs weighing from 12 to 15 kilos. The results were always the same.

The two graphs listed as Figs. 2 and 3 show the effect of injecting histamine into the saphenous and large mesenteric veins

⁷ Tatum, A. L., and Parsons, E., *J. Lab. and Clin. Med.*, 1922-23, viii, 64.

⁸ Woodyatt, R. T., *J. Biol. Chem.*, 1917, *xxix*, 355.



FIG. 1.



FIG. 2.

of a dog weighing 22.6 kilos. A perfectly definite fall in blood pressure was obtained when the histamine was injected at the rate of 0.0023 mg. per minute per kilo body weight. The fall in blood pressure was very decided when the rate was increased to 0.00048 mg. per minute per kilo body weight. The rate of respiration was slightly increased. When the histamine solution was injected into one of the large mesenteric veins at the rate of



FIG. 3.



FIG. 4.

0.011 mg. per minute per kilo body weight, a decided fall in blood pressure was obtained. The effect became more marked as the rate of injection was increased until, at the rate of 0.155 mg. per minute per kilo body weight, the animal died.

From this experiment it is apparent that histamine evokes considerable systemic action even when it is forced to pass through the liver before it passes into the general circulation.

Fig. 4 is a summary of results, obtained on one animal, that illustrates what we have found to be invariably true in a large number of experiments. In this case the rate of injection was

constant, namely 0.006 mg. per minute per kilo body weight. The most decided effect is always obtained when the histamine is injected directly into the systemic circulation (saphenous vein). *When the amine is so injected that it has to pass through a capillary network before reaching the general circulation, its effect is reduced. This is just as true of the capillaries of the lower extremity (injection into femoral artery) as it is of the capillaries of the liver.* When histamine is injected at the rate of 0.006 mg. per minute per kilo body weight into either the mesenteric or the splenic vein, from which the blood passes through the liver before it becomes a part of the general circulation, a marked systemic effect results, which is little less than that obtained when the amine is injected into the saphenous vein. The liver can, therefore, not be considered as an organ which takes a large part in the effective detoxication of histamine.

The introduction of histamine, 100 mg., into the duodenum, does not cause the slightest systemic reaction.

DISCUSSION.

It might be well, at this time, to summarize briefly all our results that might have a bearing upon intestinal intoxication.

The large intestine of man has been found normally to contain microorganisms that have the faculty of decarboxylating histidine to histamine and tyrosine to tyramine. The decarboxylase activity is, probably, far more general in its extent, and future work may show that the mixture of microorganisms contained in feces has the faculty of producing toxic amines from a large number of carboxylated amino acids. These facts would lead one to expect that the intestinal contents must normally contain toxic amines. We have succeeded in demonstrating the presence of fairly large amounts of histamine in cecal contents and in normal human feces.

Guinea pigs and dogs can be fed enormous quantities of histamine without eliciting a marked systemic reaction, if the amine is introduced directly into the stomach. The histamine so ingested is not destroyed while it remains in the stomach or in the lumen of the intestine. Over half of the amine is absorbed within 2 hours in the case of either animal. A small fraction thereof

appears in the intestinal wall and a small amount is also present in the liver. From this we can conclude that some of the amine is absorbed as such. If all the histamine that disappeared from the alimentary tract was absorbed and cast into the general circulation this, in our experiments, would be at the rate of 0.5 mg. per minute for the guinea pig and 2.2 mg. per minute for the dog. This would certainly lead to the death of the guinea pig and would, probably, eventually kill the dog. The guinea pig, however, was only mildly ill and the dog showed no symptoms of any kind.

When 100 mg. of histamine is introduced into the duodenum of a dog, the systemic blood pressure remains unaffected. If the injection is made into the saphenous vein at the rate of 0.0027 mg. per minute per kilo body weight the blood pressure is perceptibly reduced. A very pronounced fall in blood pressure is obtained when the injection rate is increased to 0.0054 mg. per minute per kilo body weight. This same injection rate is less effective, but still markedly so, when the injections are made so that the amine has to traverse a capillary network before it passes into the systemic circulation. The capillary network acts as a buffer. The capillaries of the liver seem no more effective than those of the lower extremity. In short, the liver does not destroy or modify any considerable amount of histamine so that it no longer evokes its typical pharmacological activity.

Additional evidence for this conclusion that the liver plays only a very subordinate rôle in the detoxication of histamine is contained in recent work of Dr. A. C. Ivy⁹ of the Department of Physiology at the University of Chicago. Dr. Ivy could introduce into the stomach of dogs, which had Eck fistulas, from 500 to 750 mg. of histamine (dissolved in 100 cc. of water) without producing any symptoms of intoxications. These dogs behaved like our normal dogs.

We have here to deal with a most remarkable phenomenon. Introduced into the lumen of the intestine, histamine disappears very rapidly without evoking a marked pharmacological reaction. When injected just outside of the intestinal wall, en route to the liver, a marked reaction is obtained. We are forced, therefore, to draw the inference that histamine may be rendered pharma-

⁹ Personal communication.

cologically inert in its passage through the wall of the intestine. We are now attempting to devise experiments that will further illuminate this situation.

If, for the sake of the argument, we make the assumption that histamine is modified in its passage through the intestinal wall, all our experiments are easily explicable. In the less susceptible dog the absorption was sufficiently slow, per unit area of intestine, so that what histamine did escape modification was insufficient to evoke systemic symptoms. In the much smaller guinea pig, sufficient of the amine escaped modification to produce mild symptoms of histamine intoxication.

On this assumption, injury to the intestine might, under suitable conditions, lead to grave symptoms. Meakins and Harington produced an injury to the intestines by cutting off the blood supply. This, of course, converts the intestinal wall from a living absorption area into a dead semipermeable membrane. It is not surprising that they found that this *reduced* rather than augmented the absorption rate.

SUMMARY.

1. When a small amount of histamine is introduced into a guinea pig's mouth, the animal dies within a few minutes.

2. The introduction of 100 mg. of histamine dichloride into the stomach of a guinea pig leads to mild symptoms of intoxication which persist for about 2 hours. The animal recovers completely.

3. At the end of 2 hours the contents of the alimentary tract contained 33.5 mg., the intestinal wall 4.7 mg., and the liver 4.5 mg. of histamine dichloride. A part of the histamine ingested has, therefore, passed through the intestinal wall as histamine. The amine absorbed, 57.3 mg., could not have passed into the circulation as such because this would be an absorption rate of 0.5 mg. per minute which would certainly have killed the guinea pig.

4. Histamine has practically disappeared from the alimentary tract of guinea pigs 24 hours after its ingestion.

5. The ingestion of 500 mg. of histamine by a dog weighing 5 kilos, does not produce symptoms of any kind.

6. 2 hours after the histamine had been ingested the stomach contained 147 mg. and the intestine, 88.6 mg. of histamine dichloride. The average absorption rate in this case was 2.2 mg. per minute. This quantity of histamine could not have passed into the systemic circulation, as such, without producing severe symptoms of intoxication.

7. A perceptible fall in blood pressure is obtained, in dogs, when histamine is injected into the saphenous vein at the rate of 0.0027 mg. per minute per kilo body weight. When injected at the rate of 0.0054 mg. per minute per kilo body weight, a very decided reaction is obtained.

8. When histamine is injected so that it has to traverse a capillary network before passing into the systemic circulation, its effectiveness is reduced but not obliterated. The capillaries of the limb are as effective as those of the liver. Histamine, when injected into the mesenteric or splenic veins, evokes its characteristic systemic reaction. The reaction is somewhat less than that obtained by way of the saphenous vein, but the reduction in effectiveness is too slight to speak of the liver as a detoxicator of histamine. We are more probably dealing here with a buffer action of the large capillary network.

9. When 100 mg. of histamine are introduced into the duodenum of a dog, the systemic blood pressure is not affected in any way.

10. The conclusion has been reached, tentatively, that histamine may be rendered pharmacologically inert in its passage through the intestinal wall.

THE SYNTHESIS OF NORMAL FATTY ACIDS FROM STEARIC ACID TO HEXACOSANIC ACID.

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The structures of the higher fatty acids occurring in nature are still unknown. The case of lignoceric acid offers a good illustration. For a long time it was considered a normal acid. However, Meyer, Brod, and Soyka¹ prepared the normal tetracosanic acid and found its physical constants differed from those of lignoceric acid. The findings of these authors were substantiated in their principal points by Levene and West.² Recently, the question of the structure of lignoceric acid was reopened by Brigl and Fuchs.³ These authors worked on the acid obtained from old beechwood tar and succeeded in separating it into two fractions; one melting at 74°C. and the other at 85°C. The latter is the melting point of the normal tetracosanic acid. Thus Brigl urges the return to the older view of the structure of lignoceric acid. The contentions of Brigl, regarding the structure of lignoceric acid, will be discussed in a subsequent paper. His work is referred to at this time principally in order to illustrate the necessity of an accurate knowledge of the physical constants of the higher normal fatty acids.

In the identification of any organic substance, a physical constant of a single derivative was never regarded as conclusive evidence, whereas in the work on higher fatty acids, the decision most frequently rested only on the melting point. The melting points of the esters as a rule are still less to be relied upon than those of the free acids. In a previous publication on the α -hy-

¹ Meyer, H., Brod, L., and Soyka, W., *Monatsh. Chem.*, 1913, xxxiv, 1113.

² Levene, P. A., and West, C. J., *J. Biol. Chem.*, 1914, xviii, 477.

³ Brigl, P., and Fuchs, E., *Z. physiol. Chem.*, 1922, cxix, 280.

droxy pentacosanic acid, a more thorough way for identification of the higher fatty acids was pointed out by us. This consisted in a gradual degradation of a higher fatty acid into a series of lower homologues and of a comparison of their physical constants with those of the corresponding synthetic acids. It is known that the differences in the melting points of consecutive homologous fatty acids diminish with the increasing number of carbon atoms in the chain and, hence, the evidence of a degradation product becomes gradually more convincing as the carbon atoms diminish in number. For this purpose, a new series of normal fatty acids, beginning with stearic acid and leading up to hexacosanic acid, was prepared. In the purification of fatty acids Brigl introduced the use of pyridine. This reagent was found useful in many instances; hence, it was employed as a matter of routine in the purification of every acid here described. It will be seen from Figs. 1 to 6 that there is reason to believe that the melting points reported in the present paper may be considered more reliable than the older ones (whenever there is a discrepancy) inasmuch as they fall on a smoother curve.

The syntheses of the acids differed from the one employed previously by the present writers and also by Brigl in that the higher homologues were prepared not by way of the malonic esters but through the cyanides. This process led to purer products and, besides, made possible the preparation not only of the fatty acids with an even number of carbon atoms but also those with an odd number. The complete process was as follows:



There is a theoretical point in the paper of Brigl which deserves special consideration. Brigl points out that two fatty acids of identical structure may differ in their melting points depending on the different orientation of the carbon atoms in the crystal. If such isomerism exists, one would expect it to be dynamic and it is possible that one form may be more stable than the other. If the reorientation is spontaneous and occurs continually, it should come into evidence more clearly with the age of the original substance. Indeed, it has been observed for some time in our laboratory that the melting points of fatty acids and of hydrocarbons were lowered if kept for several years in specimen bottles. From

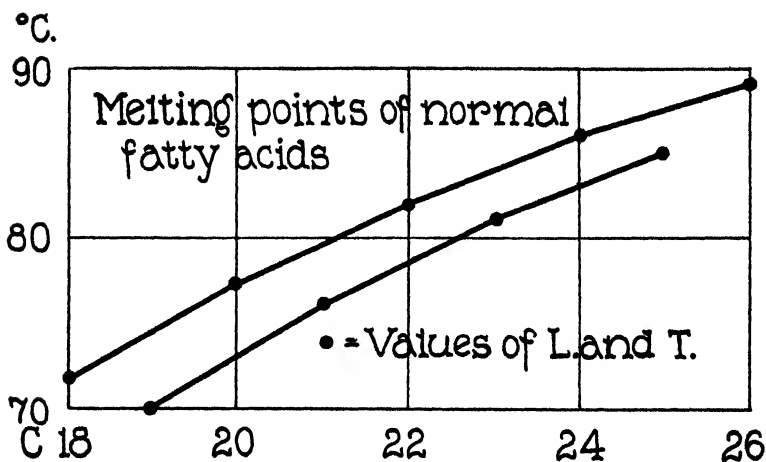


FIG 1.

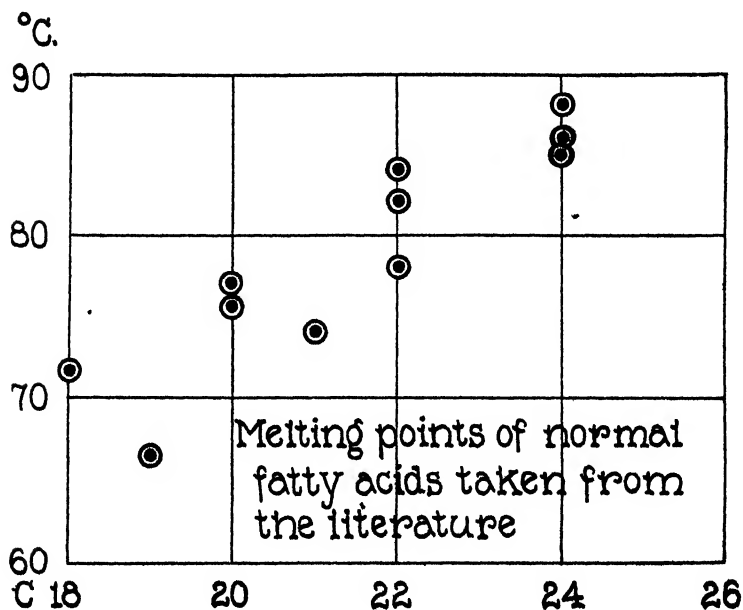


FIG 2

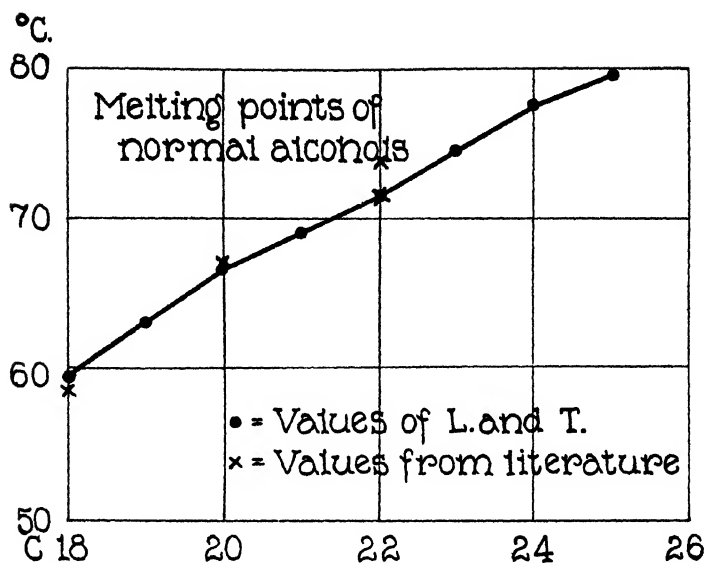


FIG. 3.

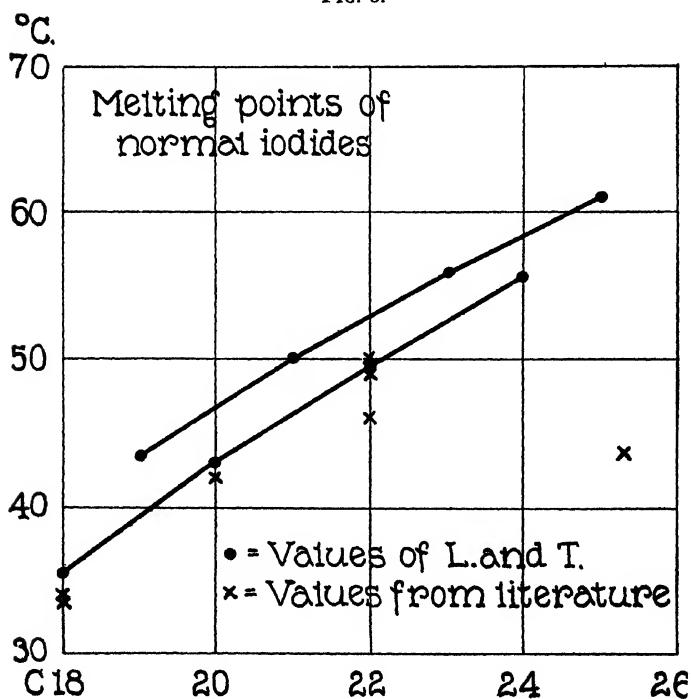


FIG. 4.

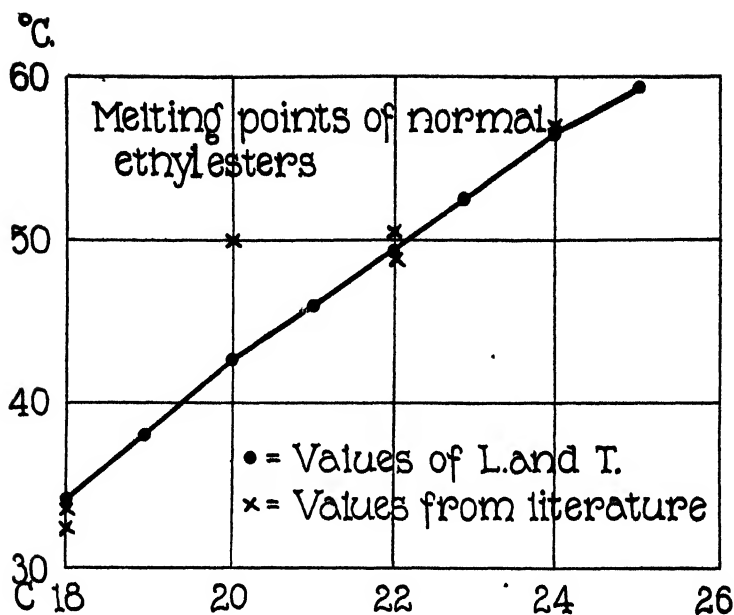


FIG. 5.

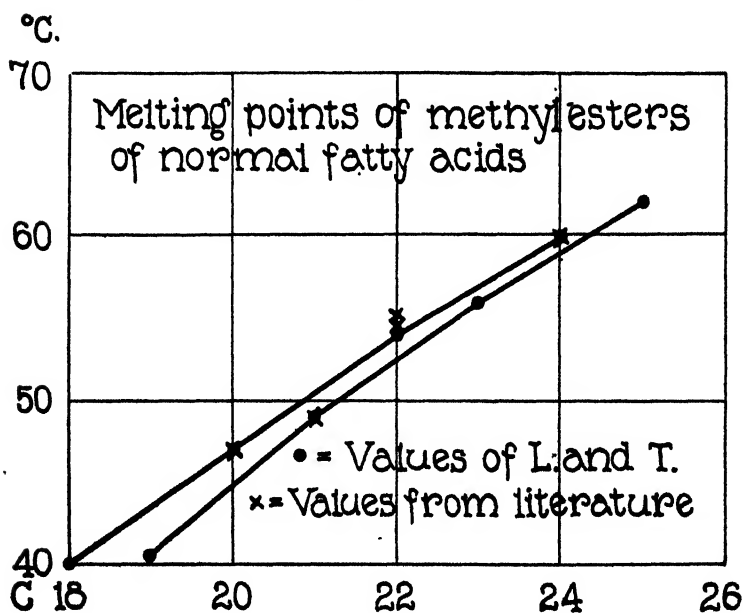


FIG. 6.

such altered, material substances with the original melting point could be recovered by recrystallization. Frequently, however, the proportion of the original substance recovered was very small. The practical conclusion from this observation is that work dealing with the melting points of the derivatives of the higher hydrocarbons must take into consideration the age of the materials. This point will be discussed in greater detail in a subsequent paper.

EXPERIMENTAL PART.

The starting points for the two series of syntheses to be described were Kahlbaum's oleic and erucic acids.

The oleic acid was reduced to stearic acid and the latter built up, by the addition of one carbon atom at a time, to *n*-docosanic acid. The erucic acid was reduced to *n*-docosanic acid and this converted, in the same manner, into *n*-hexacosanic acid.

The oleic acid (200 gm.) was dissolved in 99.5 per cent alcohol (650 cc.), sulfuric acid (20 gm.) was added, and the solution boiled overnight. The product was diluted with ice and water and washed until the washings were no longer acid. The ester was then collected in ether, dried over sodium sulfate, and distilled. The yield was 210 gm.

The ethyl oleate (50 gm.) was reduced in solution in 99.5 per cent alcohol (2,000 cc.), containing glacial acetic acid (10 cc.), by shaking in an atmosphere of hydrogen in the presence of colloidal palladium (0.1 gm.). When the absorption of hydrogen had practically ceased, the solution was cooled to 0°C. The stearic ester which separated was recrystallized from 99.5 per cent alcohol and distilled.

Ethyl *n*-docosanate was prepared and purified in an exactly analogous manner.

The other ethyl esters were prepared by dissolving the corresponding acids in about 10 parts of 99.5 per cent alcohol, containing 2 per cent of sulfuric acid, and boiling the solutions overnight. The esters crystallized at 0°C. They were crystallized twice more—once from alcohol and once from acetone—and distilled. Middle fractions were again crystallized from acetone.

Ethyl Stearate.—The ester boiled at 152°C. at 0.18 mm. with the bath at 205°C. All fractions melted⁴ at 32.5–33.5°C. The middle fraction, after crystallization from acetone melted at 33–34°C. The melting points recorded in the literature vary between 32.9 and 33.7°C.

0.1001 gm. substance: 0.2823 gm. CO₂ and 0.1123 gm. H₂O.
 C₂₀H₄₀O₂. Calculated. C 76.92, H 12.82.
 Found. " 76.91, " 12.55.

Ethyl n-Nonadecanate.—The crude ester melted at 37–38°C. It boiled at 166–168°C. at 0.27 mm. with the bath at 205°C. Distillation and crystallization from acetone failed to change the melting point.

0 1000 gm. substance: 0 2833 gm. CO₂ and 0.1128 gm. H₂O.
 C₂₁H₄₂O₂. Calculated. C 77.30, H 12.89.
 Found. " 77.26, " 12.62.

Ethyl n-Eicosanate.—Both the crude and the purified samples melted at 41.5–42.5°C. while the melting point of the arachidic ethyl ester is recorded in the literature as 50°C. Recently, however, Ehrenstein and Stuewer⁵ have given evidence to show that arachidic acid is not *n*-eicosanic acid but isodocosanic acid. The ester distilled at 177°C. at 0.28 mm. with the bath at 211°C.

0 1000 gm. substance: 0.2851 gm. CO₂ and 0.1141 gm. H₂O.
 C₂₂H₄₄O₂. Calculated. C 77.65, H 12.94.
 Found. " 77.75, " 12.77.

Ethyl n-Heneicosanate.—The ester, after crystallization once from 99.5 per cent alcohol and twice from acetone, melted at 45–46°C. as did the crude substance.

0 1005 gm. substance: 0.2877 gm. CO₂ and 0.1197 gm. H₂O.
 C₂₃H₄₆O₂. Calculated. C 77.97, H 13.00.
 Found. " 78.07, " 13.32.

⁴ The melting points were determined by heating the bath at such a rate that the temperature was raised 1°C. in 6 seconds, the bath being well stirred. The temperatures recorded are those at which the substances began to liquefy at the walls of the tube and those at which they were completely liquid. After each melting point the substance was cooled 10° below the point at which it solidified and the melting point again determined. A standard Anschütz thermometer was used.

⁵ Ehrenstein, R., and Stuewer, H., *J. prakt. Chem.*, 1922–23, cv, 199.

Ethyl n-Docosanate (Behenate).—The crude ester melted at 48–49°C. It boiled at 184–185°C. at 0.20 mm. with the bath at 245°C. It was then crystallized from acetone and melted at 48.5–49.5°C. Toyama⁶ found a melting point of 50–50.5°C.

0.0999 gm. substance: 0.2864 gm. CO₂ and 0.1176 gm. H₂O.

C₂₄H₄₈O₂. Calculated. C 78.26, H 13.04.

Found. " 78.18, " 13.17.

Ethyl n-Tricosanate.—The ester first melted at 52.5–53.5°C. After distillation and crystallization from acetone, it melted at 52–53°C. It boiled at 198–199°C. at 0.27 mm. with the bath at 250°C.

0.1002 gm. substance: 0.2881 gm. CO₂ and 0.1169 gm. H₂O.

C₂₅H₅₀O₂. Calculated. C 78.54, H 13.09.

Found. " 78.41, " 13.05.

Ethyl n-Tetracosanate.—Both the crude and purified samples of the ester melted at 55.5–56.5°C. Levene, West, Allen, and van der Scheer⁷ have reported a melting point of 56–57°C. It boiled at 198–199°C. at 0.24 mm. with the bath at 246°C.

0.1000 gm. substance: 0.2889 gm. CO₂ and 0.1176 gm. H₂O.

C₂₆H₅₂O₂. Calculated. C 78.79, H 13.13.

Found. " 78.78, " 13.16.

Ethyl n-Pentacosanate.—The ester melted at 58–59°C. both before and after purification. It boiled at 216–217°C. at 0.50 mm. with the bath at 265°C.

0.1001 gm. substance: 0.2909 gm. CO₂ and 0.1186 gm. H₂O.

C₂₇H₅₄O₂. Calculated. C 79.02, H 13.17.

Found. " 79.25, " 13.26

The methyl esters were prepared by boiling the solutions of the acids in absolute methyl alcohol, containing 2 per cent of sulfuric acid, overnight. Sufficient alcohol was used to dissolve the acids. The esters were then crystallized at 0°C. and recrystallized from acetone as many times as necessary to obtain constant melting points. Usually one or two crystallizations were sufficient.

⁶ Toyama, Y., *J. Chem. Ind. Japan*, 1922, xxv, 1053.

⁷ Levene, P. A., West, C. J., Allen, C. H., and van der Scheer, J., *J. Biol. Chem.*, 1915, xxiii, 71.

Methyl Stearate.—The crude ester melted at 39–40°C. One crystallization from acetone brought the melting point to 38.5–39.5°C., after which it was not changed. Several preparations showed the same melting point. The melting point recorded in the literature is 38°C.

0.1004 gm. substance: 0.2810 gm. CO₂ and 0.1154 gm. H₂O.

C₁₈H₃₆O₂. Calculated. C 76.51, H 12.75.

Found. " 76.32, " 12.86.

Methyl n-Nonadecanate.—The melting point of the crude ester was 39.5–41°C. After crystallization from acetone it melted at 39.5–40.5°C.

0.1002 gm. substance: 0.2822 gm. CO₂ and 0.1154 gm. H₂O.

C₂₀H₄₀O₂. Calculated. C 76.92, H 12.82.

Found. " 76.80, " 12.88.

Methyl n-Eicosanate.—On crystallization from acetone, the melting point of the ester decreased from 46.5–47.5°C. to 46–47°C. Ehrenstein and Stuewer⁵ give the same melting point.

0.1001 gm. substance: 0.2830 gm. CO₂ and 0.1162 gm. H₂O.

C₂₁H₄₂O₂. Calculated. C 77.30, H 12.88.

Found. " 77.10, " 12.99.

Methyl n-Heneicosanate.—The crude ester melted at 47.5–48.5°C. Crystallization from acetone brought it to 48–49°C. where it was constant. Le Sueur and Withers⁸ found the same melting point.

0.0998 gm. substance: 0.2842 gm. CO₂ and 0.1174 gm. H₂O.

C₂₂H₄₄O₂. Calculated. C 77.65, H 12.94.

Found. " 77.65, " 13.16.

Methyl n-Docosanate (Behenate).—The crude ester melted at 53–54°C. It was not changed by crystallization from acetone. Meyer, Brod, and Soyka¹ and Toyama⁶ record melting points of 55°C. and 54–54.5°C., respectively.

0.1003 gm. substance: 0.2863 gm. CO₂ and 0.1200 gm. H₂O.

C₂₃H₄₆O₂. Calculated. C 77.97, H 13.00.

Found. " 77.84, " 13.39.

Methyl n-Tricosanate.—In this case also the melting point of the crude ester was the same as that of the purified sample, 55–56°C.

⁸ Le Sueur, H. R., and Withers, J. C., *J. Chem. Soc.*, 1915, cvii, 736.

0.1003 gm. substance: 0.2883 gm. CO_2 and 0.1191 gm. H_2O .

$\text{C}_{24}\text{H}_{48}\text{O}_2$. Calculated. C 78.26, H 13.04.

Found. " 78.38, " 13.29.

Methyl n-Tetracosanate.—The melting point of this ester was 59–60°C. while Meyer, Brod, and Soyka¹ have reported 59.5–60°C.

0.1002 gm. substance: 0.2880 gm. CO_2 and 0.1213 gm. H_2O .

$\text{C}_{24}\text{H}_{48}\text{O}_2$. Calculated. C 78.54, H 13.09.

Found. " 78.38, " 13.54.

Methyl n-Pentacosanate.—Crude and purified samples of the ester melted at 61–62°C.

0 1001 gm. substance: 0.2895 gm. CO_2 and 0.1211 gm. H_2O .

$\text{C}_{25}\text{H}_{50}\text{O}_2$. Calculated. C 78.79, H 13.13.

Found. " 78.88, " 13.54.

The alcohols were prepared by the method described by Levene and Cretcher⁹ and Levene and Taylor¹⁰ for the reduction of ethyl esters by sodium and 99.5 per cent alcohol. The lower members were reduced in 15 gm. lots while in the case of the higher ones, because of a decreased solubility, 10 gm. lots were more convenient. The yield of alcohol was about 80 per cent of the theoretical. The remaining 20 per cent was largely recovered as fatty acid which could be again esterified and reduced to give rise to pure alcohol.

The alcohols were extracted from the admixed fatty acid sodium salt with boiling acetone, recrystallized from acetone at 0°C., and usually distilled. They were then again crystallized from acetone at 0°C. Generally the crude samples melted within 0.5°C. of the purified substances.

n-Octadecanol.—The alcohol melted at 58.5–59.5°C. Levene, West, and van der Scheer¹¹ and Gascard¹² have previously reported 58.5°C. It boiled at 153–154°C. at 0.27 mm. with the bath at 185–190°C.

⁹ Levene, P. A., and Cretcher, L. H., Jr., *J. Biol. Chem.*, 1918, xxxiii, 505.

¹⁰ Levene, P. A., and Taylor, F. A., *J. Biol. Chem.*, 1922, lii, 227.

¹¹ Levene, P. A., West, C. J., and van der Scheer, J., *J. Biol. Chem.*, 1915, xx, 521.

¹² Gascard, A., *Ann. chim.*, 1921, xv, 332.

0.1002 gm. substance: 0.2939 gm. CO_2 and 0.1235 gm. H_2O .

$\text{C}_{18}\text{H}_{38}\text{O}$. Calculated. C 80.00, H 14.07.

Found. " 79.99, " 13.79.

n-Nonadecanol.—It melted at 62–63°C. and boiled at 166–167°C. at 0.32 mm. with the bath at 195°C.

0.1003 gm. substance: 0.2949 gm. CO_2 and 0.1252 gm. H_2O .

$\text{C}_{19}\text{H}_{40}\text{O}$. Calculated. C 80.28, H 14.08.

Found. " 80.18, " 13.97.

n-Eicosanol.—Both the crude and purified samples melted at 65.5–66.5°C. Levene, West, and van der Scheer¹¹ obtained a melting point of 66–67°C. The alcohol boiled at 178°C. at 0.40 mm. with the bath at 217°C.

0.1004 gm. substance: 0.2971 gm. CO_2 and 0.1286 gm. H_2O .

$\text{C}_{20}\text{H}_{42}\text{O}$. Calculated. C 80.53, H 14.09.

Found. " 80.70, " 14.33.

n-Heneicosanol.—This alcohol melted at 68–69°C.

0.0996 gm. substance: 0.2952 gm. CO_2 and 0.1268 gm. H_2O .

$\text{C}_{21}\text{H}_{44}\text{O}$. Calculated. C 80.77, H 14.10.

Found. " 80.82, " 14.24.

n-Docosanol.—The crude alcohol melted at 71–72°C. Purification caused the melting point to decrease to 70.5–71.5°C. It boiled at 180°C. at 0.22 mm. with the bath at 230°C. Willstätter and Mayer¹² and Levene, West, Allen, and van der Scheer⁷ have reported melting points of 71–71.5°C. and 73–74°C., respectively.

0.1001 gm. substance: 0.2966 gm. CO_2 and 0.1286 gm. H_2O .

$\text{C}_{22}\text{H}_{46}\text{O}$. Calculated. C 80.98, H 14.11.

Found. " 80.80, " 14.38.

n-Tricosanol.—The melting point is 73.5–74.5°C. and the boiling point 191–193°C. at 0.7 mm. with the bath at 245–250°C.

0.1000 gm. substance: 0.2985 gm. CO_2 and 0.1267 gm. H_2O .

$\text{C}_{23}\text{H}_{48}\text{O}$. Calculated. C 81.18, H 14.12.

Found. " 81.40, " 14.18.

n-Tetracosanol.—The crude alcohol melted at 76–77°C. After crystallization and distillation it melted at 76.5–77.5°C. It boiled at 210–210.5°C. at 0.40 mm. with the bath at 240–245°C. Brigl and Fuchs⁸ have reported a melting point of 77.5°C.

¹² Willstätter, R., and Mayer, E. W., *Ber. chem. Ges.*, 1908, xli, 1478.

0 1001 gm. substance: 0.2998 gm. CO_2 and 0.1266 gm. H_2O .

$\text{C}_{24}\text{H}_{50}\text{O}$. Calculated. C 81.36, H 14.12.

Found. " 81.67, " 14.15.

n-Pentacosanol.—The purified alcohol melted, 0.5°C . higher than the crude material, at 78.5 – 79.5°C . It boiled at 214 – 216°C . at 0.36 mm. with the bath at 260°C .

0 0999 gm. substance: 0 2978 gm. CO_2 and 0.1272 gm. H_2O .

$\text{C}_{25}\text{H}_{52}\text{O}$. Calculated. C 81.52, H 14.13.

Found. " 81.29, " 14.25.

The alcohols were converted into the iodides by heating at 180°C . for an hour with 1.1 equivalents of iodine and an excess of red phosphorus. The iodides were then crystallized from acetone until the melting points were constant. Some of them were distilled.

n-Octadecyl Iodide.—The crude iodide melted at 34 – 35°C . Distillation and crystallization from acetone raised the melting point to 34.5 – 35.5°C . It boiled at 164°C . at 0.22 mm. with the bath at 208°C . Gascard¹² found a melting point of 33.5°C . and Levene, West, and van der Scheer¹¹ have reported 34°C .

0 2036 gm. substance: (Carius) 0.1250 gm. AgI.

$\text{C}_{18}\text{H}_{37}\text{I}$. Calculated. I 33 42.

Found. " 33.14.

n-Nonadecyl Iodide.—The iodide melted at 42.5 – 43.5°C . and boiled at 174 – 174.5°C . at 0.22 mm. with the bath at 214 – 217°C .

0 1015 gm. substance: (Carius) 0 0604 gm. AgI.

$\text{C}_{19}\text{H}_{39}\text{I}$. Calculated. I 32.23.

Found. " 32.16.

n-Eicosyl Iodide.—The melting point of this iodide is 42 – 43°C . Levene, West, and van der Scheer¹¹ have reported 42°C .

0.1010 gm. substance: (Carius) 0.0584 gm. AgI.

$\text{C}_{20}\text{H}_{41}\text{I}$. Calculated. I 31.13.

Found. " 31.24.

n-Heneicosyl Iodide.—The melting point is 49 – 50°C .

0 1011 gm. substance: 0 0563 gm. AgI.

$\text{C}_{21}\text{H}_{43}\text{I}$. Calculated. I 30.09.

Found. " 30.09.

n-Docosyl Iodide.—It melted at 48.5–49.5°C. Meyer, Brod, and Soyka,¹ Levene, West, and van der Scheer¹¹ and Brigl¹⁴ found melting points of 46, 49, and 48.5–50°C., respectively. The boiling point is 198°C. at 0.23 mm. with the bath at 245°C.

0.1004 gm. substance: (Carius) 0.0548 gm. AgI.
 $C_{22}H_{44}I$. Calculated. I 29.13.
Found. " 29.50.

n-Tricosyl Iodide.—Both crude and purified samples melted at 55–56°C. It boiled at 205°C. at 0.45 mm. with the bath at 250°C.

0.2008 gm. substance: (Carius) 0.1038 gm. AgI.
 $C_{23}H_{46}I$. Calculated. I 28.22.
Found. " 27.94.

n-Tetracosyl Iodide.—The melting point of the crude material was raised 0.5°C. by purification, to 54.5–55.5°C. It boiled at 207–209°C. at 0.35 mm. with the bath at 272–275°C.

0.1024 gm. substance: (Carius) 0.0516 gm. AgI.
 $C_{24}H_{48}I$. Calculated. I 27.37.
Found. " 27.23.

n-Pentacosyl Iodide.—This iodide melted at 60–61°C.

0.1011 gm. substance: (Carius) 0.0508 gm. AgI.
 $C_{25}H_{50}I$. Calculated. I 26.55.
Found. " 27.15.

The iodides were dissolved in 10 to 15 parts of 99.5 per cent alcohol together with an excess of potassium cyanide and boiled overnight. The corresponding cyanides separated on cooling. They were crystallized from acetone at 0°C. until the melting points were constant and the inorganic matter had been removed.

n-Octadecyl Cyanide.—The melting point is 42.5–43.5°C.

0.2000 gm. substance required 7.15 cc. 0.1 N HCl.
 $C_{18}H_{35}N$. Calculated. N 5.02.
Found. " 5.00.

n-Nonadecyl Cyanide.—It melted at 49.5–50.5°C.

0.2000 gm. substance required 6.85 cc. 0.1 N HCl.
 $C_{20}H_{39}N$. Calculated. N 4.78.
Found. " 4.80.

¹⁴ Brigl, P., *Z. physiol. Chem.*, 1915, xcv, 161.

n-Eicosyl Cyanide.—This cyanide melted at 48.5–49.5°C. and solidified at 42.5°C. After cooling 10° further and then remelting, it melted at 43.5–44.5°C. Only after standing for a time at room temperature did it again have the higher melting point.

0.2000 gm. substance required 6.60 cc. 0.1 N HCl.

$C_{21}H_{41}N$. Calculated. N 4.56.

Found. " 4.62.

n-Heneicosyl Cyanide.—Again two melting points were obtained. It first melted at 55.5–56.5°C. Then, after cooling 10° below its solidification point, it melted at 51.5–52.5°C. After standing at room temperature it again had the higher melting point.

0.2026 gm. substance required 6.25 cc. 0.1 N HCl.

$C_{22}H_{43}N$. Calculated. N 4.36.

Found. " 4.31.

n-Docosyl Cyanide.—It first melted at 53.5–54.5°C. It solidified at 50.5°C. and, after cooling 10° and reheating, melted at 51.5–52.5°C.

0.2000 gm. substance required 5.85 cc. 0.1 N HCl.

$C_{23}H_{45}N$. Calculated. N 4.18.

Found. " 4.09.

n-Tricosyl Cyanide.—Two melting points were also obtained with this substance, the first at 61–62°C. and the second at 55–56°C.

0.2000 gm. substance required 5.65 cc. 0.1 N HCl.

$C_{24}H_{47}N$. Calculated. N 4.01.

Found. " 3.96.

n-Tetracosyl Cyanide.—This cyanide melted and remelted at 58–59°C.

0.2000 gm. substance required 5.25 cc. 0.1 N HCl.

$C_{25}H_{49}N$. Calculated. N 3.86.

Found. " 3.67.

n-Pentacosyl Cyanide.—The melting point is 61–62°C.

0.2011 gm. substance required 4.85 cc. 0.1 N HCl.

$C_{26}H_{51}N$. Calculated. N 3.71.

Found. " 3.38.

The fatty acids were prepared by boiling the alcoholic solutions of the cyanides with sodium hydroxide overnight. Usually a large excess of 50 per cent sodium hydroxide solution was added to the solution in which the cyanide was formed and the saponification carried out without the isolation of the cyanide. The sodium salts were then filtered from the cooled solutions and washed with alcohol, acetone, and water (to remove potassium cyanide).

The acids were liberated by heating the salts with dilute hydrochloric acid on the water bath until clear, transparent oils were obtained. Crystallization from acetone at 0°C. usually gave acids with the correct melting points. The yields of acids from the iodides were 93 to 96 per cent of the theoretical.

The samples of acid for characterization were prepared from the distilled ethyl esters. After saponification, they were crystallized from acetone and from pyridine. The least soluble fractions from pyridine were melted over dilute hydrochloric acid on the water bath and again crystallized from acetone.

In addition, the lead salts were precipitated from boiling methyl alcohol, filtered off, and washed with acetone and with water. They were then dried, dissolved in hot toluene, and the acids liberated by passing hydrogen sulfide through the solutions.

Acid numbers are reported as molecular weights. The sample of acid was dissolved in 25 cc. of toluene and 50 cc. of methyl alcohol, the solution heated to the boiling point and titrated with 0.1 N sodium hydroxide, using phenolphthalein as indicator.

Stearic Acid.—The acid was prepared by the saponification of the distilled ethyl ester from the reductions of ethyl oleate. It melted at 70.5–71.5°C. Crystallization from pyridine and conversion into the lead salt with subsequent liberation of the acid failed to alter it. The highest melting point recorded in the literature is 71–71.5°C. by Saizew.¹⁵

0.0998 gm. substance: 0.2780 gm. CO₂ and 0.1138 gm. H₂O.

0.6045 " " required 21.20 cc. 0.1 N NaOH.

C₁₈H₃₆O₂. Calculated. C 76.06, H 12.70, Mol. wt. 284.

Found. " 75.96, " 12.76, " " 285.

¹⁵ Saizew, *J. Russ. Phys. Chem. Soc.*, 1885, xvii, 425.

n-Nonadecanic Acid.—The crude acid from the cyanide melted at 69–70°C. Purification failed to change it. Schweizer¹⁶ has reported a melting point of 66.5°C.

0 1002 gm. substance: 0 2805 gm. CO₂ and 0 1163 gm. H₂O.
 0 5971 “ “ required 20 12 cc. 0.1 N NaOH.
 C₁₉H₃₈O₂. Calculated. C 76.51, H 12.75, Mol. wt. 298.
 Found. “ 76.34, “ 12.99, “ “ 297.

n-Eicosanic Acid.—In this case also the crude and purified samples melted at the same temperature, 76–77°C. Schweizer¹⁶ and Levene, West, and van der Scheer¹¹ have reported melting points of 75.5 and 77°C., respectively.

0 1000 gm. substance: 0 2816 gm. CO₂ and 0 1157 gm. H₂O.
 0 6237 “ “ required 19.80 cc. 0.1 N NaOH.
 C₂₀H₄₀O₂. Calculated. C 76.92, H 12.82, Mol. wt. 312.
 Found. “ 76.79, “ 12.95, “ “ 315.

n-Heneicosanic Acid.—It melted at 75–76°C. both before and after purification. Le Sueur and Withers⁸ found a melting point of 73–74°C.

0.1003 gm. substance: 0 2849 gm. CO₂ and 0 1167 gm. H₂O.
 0 6416 “ “ required 19 62 cc. 0.1 N NaOH.
 C₂₁H₄₂O₂. Calculated. C 77.30, H 12.88, Mol. wt. 326.
 Found. “ 77.46, “ 13.02, “ “ 327.

n-Docosanic (Behenic) Acid.—This acid was prepared from two sources. The crude acid (A) from *n*-heneicosyl cyanide melted at 80–81°C. Crystallization from pyridine brought the melting point to 81–82°C.

The other acid (B) was prepared by the saponification of the distilled ethyl ester from the reduction of ethyl erucate. The crude acid melted at 80.5–81.5°C. It was crystallized from pyridine and passed over the lead salt and then melted at 81–82°C. The melting point of a mixture of approximately equal parts of (A) and (B) was 81–82°C. Stohmann and Langbein,¹⁷ Power and Salway,¹⁸ and Meyer, Brod, and Soyka¹ have reported melting points of 77–78°C., 80–82°C., and 84°C., respectively.

¹⁶ Schweizer, A., *Arch. Pharm.*, 1884, xxii, series 3, 770.

¹⁷ Stohmann, F., and Langbein, H., *J. prakt. Chem.*, 1890, xlii, series 2, 879.

¹⁸ Power, F. B., and Salway, A. H., *J. Am. Chem. Soc.*, 1908, xxx, 256.

(A) 0.1000 gm. substance:	0.2852 gm. CO ₂ and 0.1172 gm. H ₂ O.
0.6490 " "	required 18.90 cc. 0.1 N NaOH.
(B) 0.1001 " "	: 0.2854 gm. CO ₂ and 0.1155 gm. H ₂ O.
0.7058 " "	required 20.74 cc. 0.1 N NaOH.
C ₂₃ H ₄₄ O ₂ .	Calculated. C 77.65, H 12.94, Mol. wt. 340.
	Found (A) " 77.77, " 13.11, " " 343.
	" (B) " 77.75, " 12.91, " " 340.

n-Tricosanic Acid.—The crude acid melted at 80–81°C. It was not changed by purification.

0.1004 gm. substance:	0.2866 gm. CO ₂ and 0.1169 gm. H ₂ O.
0.6969 " "	required 19.67 cc. 0.1 N NaOH.
C ₂₃ H ₄₆ O ₂ .	Calculated. C 77.97, H 13.00, Mol. wt. 354.
	Found. " 77.84, " 13.03, " " 354.

n-Tetracosanic Acid.—The melting point of the crude acid was 84.5–85.5°C. The material obtained from the distilled ethyl ester, after crystallization from pyridine, was passed over the lead salt and then melted at 85–86°C. Meyer, Brod, and Soyka¹ obtained a melting point of 85.5–86°C., while Brigl¹⁴ and Levene, West, Allen, and van der Scheer⁷ have reported 85°C. and 87.5–88°C., respectively.

0.1001 gm. substance:	0.2880 gm. CO ₂ and 0.1187 gm. H ₂ O.
0.7664 " "	required 20.70 cc. 0.1 N NaOH.
C ₂₄ H ₄₈ O ₂ .	Calculated. C 78.26, H 13.04, Mol. wt. 368.
	Found. " 78.46, " 13.27, " " 370.

n-Pentacosanic Acid.—Both the crude and purified samples melted at 84–85°C.

0.1000 gm. substance:	0.2886 gm. CO ₂ and 0.1149 gm. H ₂ O.
0.7947 " "	required 20.65 cc. 0.1 N NaOH.
C ₂₅ H ₅₀ O ₂ .	Calculated. C 78.54, H 13.09, Mol. wt. 382.
	Found. " 78.70, " 12.86, " " 385.

n-Hexacosanic Acid.—Again the melting point of the crude acid, 88–89°C., was not changed by purification.

0.1000 gm. substance:	0.2888 gm. CO ₂ and 0.1188 gm. H ₂ O.
0.7954 " "	required 20.00 cc. 0.1 N NaOH.
C ₂₆ H ₅₂ O ₂ .	Calculated. C 78.79, H 13.13, Mol. wt. 396.
	Found. " 78.76, " 13.29, " " 398.

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**PROCEEDINGS OF THE AMERICAN SOCIETY OF
BIOLOGICAL CHEMISTS.**

EIGHTEENTH ANNUAL MEETING.

St. Louis, Mo., December 27-29, 1923.

THE PRODUCTION AND PRESENCE OF POISONOUS AMINES IN THE MAMMALIAN ORGANISM.

By KARL K. KOESSLER AND MILTON T. HANKE.

(From the Otho S. A. Sprague Memorial Institute and Department of Pathology, University of Chicago, Chicago.)

Amine-producing microorganisms, *i.e.* such microorganisms which decarboxylate histidine to histamine and tyrosine to tyramine, are constant normal inhabitants of the human intestinal tract.

This decarboxylase activity is a specific function of certain strains within the same species; microorganisms which decarboxylate histidine do not decarboxylate tyramine, and *vice versa*.

Decarboxylation of the amino acids, histidine and tyrosine, and probably of other amino acids, is a normal pathway in the catabolism of proteins which occurs side by side with deamination.

Histamine and probably also tyramine, is a normal constituent of the cecal and fecal matter of man; *i.e.*, of the large intestine.

In the detoxication of histamine the liver seems to play only a very subordinate part.

The conclusion is reached, tentatively, on the basis of experimental work, that histamine is rendered pharmacologically inert in its passage through the intestinal wall by the cellular activity of the intestinal mucosa itself.

THE SOLUBILITY OF COAGULATED PROTEINS AS INDICATED BY IMMUNOLOGICAL METHODS.

By H. GIDEON WELLS AND JULIAN H. LEWIS.

(From the Otho S. A. Sprague Memorial Institute and Department of Pathology, University of Chicago, Chicago.)

Immunological methods furnish a far more delicate means of determining the presence and character of minute quantities of proteins in solution than chemical analytic methods. By means of

anaphylaxis and complement fixation reactions a study has been made of the solubility of egg white and horse serum proteins after complete coagulation by heat and removal of uncoagulated proteins by washing. It was found that coagulated egg white suspended in chloroform-water for long periods of time comes occasionally, but not always, to contain barely sufficient dissolved egg protein in 5 cc. samples to sensitize guinea pigs to egg albumin and egg globulin, as well as to egg white. The amount of whole protein in such a solution is probably something less than 0.001 mg. and probably more than one-twentieth of this amount. Coagulated horse serum is somewhat more soluble than coagulated egg white, 5 cc. doses of the supernatant fluid often containing sufficient dissolved antigenic protein to sensitize fatally, probably about 0.001 mg. or slightly more. Complement fixation tests corroborate the anaphylaxis tests and demonstrate that the antigenic protein is actually in solution. Specificity tests indicate that the protein so redissolved is the same protein that was originally coagulated; therefore, the coagulation of protein by heat is to a slight extent a true reversible reaction. Coagulated egg and serum proteins suspended in a finely divided state and injected intraperitoneally into guinea pigs undergo sufficient solution after injection to sensitize the animals to the same uncoagulated protein. Usually the sensitization obtained in this way is greater than when the protein is dissolved in water *in vitro*. Probably the reaction or salt content of the body fluids is more favorable to solution of the coagulated proteins than is pure water, but it is possible that partial enzymatic hydrolysis of the coagulated proteins in the body fluids facilitates solution of the minute amounts of antigen necessary for sensitization.

A PHYSICOCHEMICAL METHOD OF CHARACTERIZING PROTEINS. V.

By EDWIN JOSEPH COHN.

(From the Department of Physical Chemistry in the Laboratories of
Physiology, Harvard Medical School, Boston.)

In 1858 Denis described a class of proteins in the blood and in certain seeds that were soluble in salt solutions. This class of

proteins, the globulins, has since been found to have a wide distribution, but no satisfactory explanation¹ of their unique relations to electrolytes has thus far been proposed. Such an explanation is now possible as a result of the extensive investigations of Noyes and Harkins and their coworkers, and more recently of Brönsted upon the solubility in salt solutions of compounds of different valence types. Their results indicate that the solubility of slightly soluble compounds is increased by the presence of salts without a common ion and that this solvent action is greater the higher the valence of the saturating compound. We find that the solvent action of salts upon globulins is only slightly greater than that upon the tri-trivalent metal ammonia compounds studied by Brönsted. We therefore conclude that the great solvent action of salts upon globulins depends upon the high valency of these proteins at their isoelectric points.

II.

Recent experiments indicate that salts also increase the solubility of other slightly soluble proteins. The extent to which the solubility of casein is increased by very low concentrations of salt suggests that this protein must be considered either a bivalent, or a uni-trivalent compound.

Brönsted has employed the following equations for the change in solubility from S_0 to S_1 , or S_1 to S_2 , brought about by an increase in the concentration of total salt:²

$$\log \frac{S_1}{S_0} = \alpha (c_1^\beta - S_0^\beta) \text{ or } \log \frac{S_2}{S_1} = \alpha (c_2^\beta - c_1^\beta)$$

Both α and the exponential β are here constants. This equation is closely related to the empirical law for the lowering of the freezing point proposed by G. N. Lewis: $(1-Y) = \alpha c^\beta$. The equations differ chiefly in the last term, employed by Brönsted to designate the stationary state of solubility. The constants have

¹ The suggestion of Hardy that globulins form complex compounds with salts, although probably correct, offers no explanation of the unique properties of these proteins, since other proteins also possess the same chemical groups as the globulins.

² I am indebted to Professor S. P. L. Sørensen for suggesting to me, in 1920, the use of this equation in describing the effect of salts upon globulins.

approximately the same value. As a first approximation the simpler form of the equation may be used. Moreover, if the ionic strength, u , be substituted for the concentration, solvents of all valence types may be considered. Taking the logarithms of both sides we have:

$$\log \left(\log \frac{S}{S_0} \right) = \log \alpha + \beta \log u$$

If the left side of this equation be plotted as ordinate, and $\log u$ as abscissæ, β is the slope and $\log \alpha$ the value of the ordinate, when $\log u = 0$. Extrapolation yields the following constants, which are compared with the constants obtained with Brönsted's equation in Table I.

TABLE I.

Compound.	Type of valence	Method.	$\log \frac{S_0}{S} = \alpha c^\beta$		$\log \frac{S_2}{S_1} = \alpha (c_2^\beta - c_1^\beta)$	
			α	β	α	β
NaCl.....	Uni-uni.	Freezing point lowering.	(0.33)	(0.54)		
TlCl.....	"	Solubility ratio.	0.35	0.52	0.36	0.33
MgSO ₄	Bi-bi.	Freezing point lowering.	(1.44)	(0.38)		
Xantho chromate.....	"	Solubility ratio.	1.0	0.40	1.3	0.2
Casein... ..	Unknown.	" "	1.0	0.40		
Luteo hexacyano	Tri-tri.	" "	2.0	0.33	3.0	0.2
Serum globulin.....	Unknown.	" "			3.0	0.2

It should be noted that the values of α , in the Brönsted equation, increase with the square of the valence of the solute in the same way that W. B. Hardy and G. N. Lewis have shown that the ionic strength of the solvent increases with valence. These relations offer a method of investigating and determining the dissociation of such multivalent substances as the proteins.

A PHYSICOCHEMICAL METHOD OF CHARACTERIZING
PROTEINS. VI.

BY EDWIN JOSEPH COHN.

*(From the Department of Physical Chemistry in the Laboratories of
Physiology, Harvard Medical School, Boston.)*

In the previous communication¹ we have described the increase in solubility of proteins upon the addition of salt by the equation:

$$\log \frac{S}{S_0} = \alpha u\beta \quad (1)$$

This equation should hold provided solvent and solute have no common ion. In the presence of a common ion, however, the solubility product, or its function *scf*, should be introduced as the left side of the equation, as Brønsted has shown. The approximation equation then becomes:

$$\log \frac{scf}{scf_0} = \alpha u\beta \quad (2)$$

When small amounts of salt are added to casein the solubility increases, as we have reported.² Upon the addition of larger amounts of salt the solubility decreases. This can be explained if we assume that casein forms a complex compound with the salt, and that both effects of salt are then simultaneously manifest. The effect of all concentrations of salt upon casein can be described by combining equations (1) and (2).

We believe that these conceptions and equations are applicable to other proteins than casein. If proteins that are completely soluble in salt solutions have formed complex compounds, then their precipitation from stronger salt solutions is described by equation (2).

Moreover, the application of this equation to the precipitation of proteins by neutral salts offers a method of characterizing very soluble proteins, for the constant, α , in equation (2), as in equation (1), is a function of the valence of the protein.

¹ Cohn, E. J., *J. Biol. Chem.*, 1924, lix, p. iv.

POSTSCORBUTIC NUTRITION IN THE GUINEA PIG.

BY ARTHUR H. SMITH AND WILLIAM E. ANDERSON.

(From the Sheffield Laboratory of Physiological Chemistry, Yale University, New Haven.)

The purpose of the present study was to determine the effect of acute scurvy on the appetite and growth of guinea pigs during the period of recovery. Animals of the same age and of litters of the same size were employed. In each experiment two animals of the same sex were used; an experimental and a control. The diet given throughout consisted of:

	<i>per cent</i>
Soy bean meal (autoclaved)	86
Cod liver oil	5
Dried yeast	3
Calcium lactate	3
Sodium chloride	3

On this food plus 20 gm. of raw cabbage daily normal growth was obtained as determined in preliminary trials comparing the behavior on this diet with that on hay, oats, and cabbage and also with that of Minot's⁴ animals. On the soy bean food plus 0.8 gm. of dried cabbage⁵ scurvy ensued in from 13 to 16 days with animals of 150 to 200 gm. of body weight. The procedure was so planned that the experimental pig received food *ad libitum* while the control animal received only as much food as the experimental pig ate on the previous day. The effect on the growth rate of inanition *per se* was thus controlled.

The food intake of the experimental animals decreased with the onset of scurvy accompanied by a significant loss in body weight as the disease progressed. The control although suffering the same diminution of food intake as the experimental animal showed far less loss in weight which emphasizes the fact that the development of scurvy is accompanied by a definite loss of body tissue.

After the experimental animals had been given fresh cabbage they immediately resumed growth at the same rate as that of the

⁴ Minot, C. S. Senescence and rejuvenation, *J. Physiol.*, 1891, xii, 97.

⁵ The fresh cabbage was boiled in water for 1 hour and dried in an oven in air at 90–100°C. for 2 days. 0.8 gm. of cabbage thus treated was obtained from 20 gm. of the fresh leaves.

control animals. A comparison of the food intake of the two groups after the fresh cabbage had been given indicates that as the scorbutic symptoms disappeared and as normal growth was resumed, the experimental animals consumed on the average definitely more food than did the control guinea pigs.

From the above statements of the experimental results it appears that, if after severe, acute scurvy, the young guinea pig survives, subsequent growth proceeds at the normal rate accompanied, however, by a consumption of food definitely greater than that of the control animal under identical conditions.

DETERMINATIONS OF VITAMIN A.

By H. STEENBOCK, MARIANA T. NELSON, AND ARCHIE BLACK.
(*From the Department of Agricultural Chemistry, University of Wisconsin, Madison.*)

It has been current practice to determine comparative amounts of vitamin A in different foodstuffs by taking observations on the growth of rats when abundantly supplied with good protein, salts, roughage, vitamin B, and energy. From analysis of previous data comparing the incidence of ophthalmia with time of cessation of growth, it appears that a lack of the antirachitic vitamin in the absence of vitamin A may likewise be responsible for growth inhibition. This throws in doubt the value of many data of vitamin A distribution where growth failure has been used as a sole criterion of its absence. Experimentally advantage has been taken of the fact that apparently ultra-violet light has the property of substituting for the antirachitic vitamin. By its means it has been found that in many cases of so called vitamin A deficiency, growth could be reinitiated by radiation and maintained until incidence of an ophthalmia. This is taken to indicate primarily a deficiency of antirachitic vitamin rather than one of vitamin A. Using this technique a distribution of vitamin A has been studied in a number of grains and forage plants. It has been found that some millets are poor in the antirachitic vitamin but comparatively well supplied with vitamin A. The same has been found true of clover and alfalfa. Bone analyses were used for control purposes throughout.

THE USE OF THE RAT FOR THE ESTIMATION OF VITAMIN B.

BY M. H. GIVENS AND F. BEHRENDT.

(From the Research Laboratories, Northwestern Yeast Company, Chicago.)

From experiments in which old and young rats were fed and caged differently evidence has been obtained that:

The rat does not store in its body a large excess of vitamin B. This result is in accord with the findings of Steenbock and associates.⁶

The rat can supplement only slightly its intake of vitamin B by ingesting its feces. Steenbock, Sell, and Nelson⁷ say: "When rats are prevented from supplementing their diet by the consumption of excreta the vitamin B content of the experimental rations must be at least twice as high."

There does not appear to be a definite relation between the age of a rat and its duration of life on a diet devoid of vitamin B but otherwise satisfactory.

In the absence of vitamin B in its diet, the duration of life of a rat appears to depend mainly on the animal's continuously consuming this ration in an amount approaching its maintenance requirement. Initial weight seems to be secondary to this factor.

Young rats declining on a diet free of vitamin B have recovered and grown for over 300 days on a daily supplement of 50 mg. of dried yeast. These experiments show quite clearly the relation of growth to food consumption.

Litter mates, though treated exactly the same in every respect, do not always grow alike because they do not always eat alike. The rat is the one variable in feeding experiments which cannot be controlled.

The foregoing results have a direct bearing on the use of the rat for the estimation of vitamin B.

⁶ Steenbock, H., Sell, M. T., and Jones, J. H., *J. Biol. Chem.*, 1923, lv, 411.

⁷ Steenbock, H., Sell, M. T., and Nelson, E. M., *J. Biol. Chem.*, 1923, lv, 399.

QUANTITATIVE ASPECTS OF THE RELATION BETWEEN VITAMIN B AND APPETITE.

By GEORGE R. COWGILL, H. J. DEUEL, JR., AND ARTHUR H. SMITH.

(From the Sheffield Laboratory of Physiological Chemistry, Yale University, New Haven.)

A method of feeding dogs on diets consisting of isolated food substances and adequate except with respect to vitamin B was employed.⁸ Under these conditions the animals sooner or later lose all desire to eat; when a sufficient amount of some source of vitamin B is then administered, the appetite is quickly and completely restored.

We have experimented with two sources of vitamin B, namely the *Yeast Vitamin Powder (Harris)*⁹ and *Wheat Sugar*¹⁰ made from wheat embryo. Preliminary experiments in which the number of days over which a single dose of the yeast preparation was effective showed the dog's requirement of this material to be between 50 and 60 mg. per kilo of body weight per day. Long continued feeding experiments were then performed in which the Harris powder was weighed out, placed in gelatin capsules, and a capsule administered to the animal each day. 20, 30, 40, 50, and 60 mg. per kilo per day were thus tested. 20 mg. proved distinctly inadequate; the 30 mg. dose was associated with maintenance of appetite over 91 days in one case and 59 days in another. The 40, 50, and 60 mg. doses gave successful results for as long as the animals were kept on the experiment, in every case, at least 3 months. On December 5, 1923, the two animals receiving 40 mg. per kilo per day had eaten the same diet perfectly for 352 days—almost a year. The minimum vitamin B requirement of the dog when fed under these conditions, appears, in terms of the Harris powder, therefore, to be between 30 and 40 mg. per kilo of body weight per day. The accuracy of this method is not great enough to warrant a more precise statement in this regard.

The *Wheat Sugar*, after failures with smaller amounts, gave successful results in two cases over periods of approximately 7

⁸ Cowgill, G. R., *J. Biol. Chem.*, 1923, lvi, 725.

⁹ From the Harris Laboratories, Tuckahoe, N. Y.

¹⁰ From the Ward Baking Co., New York, N. Y.

and 8 months, respectively, with 0.6 gm. per kilo per day. Administration of this product was then stopped; later the animals lost their desire for food, a condition which was promptly cured by the renewed administration of this vitamin-containing product. This quite apart from its relation to the quantitative aspects of this problem, would seem to be a complete demonstration of the relation between vitamin B and appetite. When these two products are compared as to their content of vitamin B by the growing rat method, the same ratio of potencies is obtained.

THE INFLUENCE OF YEAST PRODUCT ADDITIONS TO MILK RATIONS ON THE INFERTILITY OF RATS.

By H. A. MATTILL AND C. C. CONGDON.

(From the Department of Physiology, University of Rochester, Rochester.)

The apparent improvement in reproductive function of female albino rats when yeast is added to milk rations has been found not to be due to yeast nucleic acid or to vitamin B. When hot water extracts of yeast are treated with adsorbents such as Lloyd's reagent or charcoal and the filtrate (containing Funk's vitamin D) is evaporated on starch and incorporated into milk rations to the extent of 5 or 10 per cent of the original yeast there is some improvement. This change is more marked in the case of charcoal (norit) filtrate than it is with the filtrate from Lloyd's reagent. Of eleven animals on the latter product eight showed one or more resorptions, but no births. Ovulation was regular. Of eleven animals on the norit product seven, while showing some resorptions also gave birth to ten litters, only one of which lived, two animals showed neither births nor resorptions. It would appear, therefore, since our animals on milk rations seldom, if ever, show even as much as resorption, that the charcoal filtrate provides something in the nature of Evans' vitamin X. The failure of the Lloyd's reagent filtrate can be explained by the better adsorbing qualities of this substance. Preliminary experiments on the addition of vitamin X in the form of lettuce, to milk-fed rats, indicate that this unknown is indeed one of the inadequacies of milk; but the mortality of the young is high, thus suggesting that the X factor is not the sole addition necessary to make a milk ration adequate. In male animals the variability of response to

the yeast products is much higher than in the females. In a very few cases the early degeneration of the testes so constantly found in milk-fed animals is delayed. This degeneration has been postponed but not prevented by addition of green lettuce to milk rations. Work on wheat embryo and preparations from it is in progress.

THE AVAILABILITY OF SOME POSSIBLE PRECURSORS OF LYSINE FOR LYSINE SYNTHESIS.

By HOWARD B. LEWIS, DANIEL A. MCGINTY, AND CARL S.
MARVEL.

(From the Laboratories of Physiological Chemistry, University of Michigan,
Ann Arbor, and of Organic Chemistry, University of Illinois, Urbana.)

In a previous study,¹¹ we have demonstrated that it is not possible to utilize nor-leucine in place of lysine to supply the chemical factor necessary for normal growth on diets otherwise complete but deficient in their lysine content. Similar experiments have been made with various other possible precursors, α -hydroxycaproic acid, ϵ -aminocaproic acid, and α -hydroxy- ϵ -aminocaproic acid. Young white rats were maintained on a diet in which the protein element was supplied by gliadin as follows: gliadin, 18 per cent; salt mixture (Osborne-Mendel), 4.5 per cent; starch, 50 per cent; lard, 24.5 per cent; cod liver oil, 3 per cent. Water-soluble vitamin was supplied in the form of yeast concentrate (Yeast Vitamine-Harris). With this type of diet, maintenance or very slow growth only was possible. Substitution of casein for gliadin in these diets resulted in a normal rate of growth. When any one of the caproic acid derivatives above mentioned was added to the gliadin diet, no change in the rate of growth was noted, although addition of lysine itself to the gliadin diet caused a marked increase in weight and a normal rate of growth. The failure of α -hydroxy- ϵ -aminocaproic acid to act as an available precursor of lysine is of particular interest, since it apparently demonstrates that the amination of α -hydroxy acids, observed in perfusion experiments, is not possible in the living organism, at least under the conditions of the present series of experiments.

¹¹ Lewis, H. B., and Root, L. E., *J. Biol. Chem.*, 1920, xliii, 79.

THE RELATION OF ARGININE AND HISTIDINE TO GROWTH.

BY WILLIAM C. ROSE AND GERALD J. COX.

(From the Laboratory of Physiological Chemistry, University of Illinois, Urbana.)

Comparative studies have been made of the growth of rats upon diets in which the nitrogen was supplied, respectively, by casein, completely hydrolyzed casein, and hydrolyzed casein from which arginine and histidine had been removed by the usual method of silver precipitation. Rats upon completely hydrolyzed casein grew to maturity, but at slower rates than animals of the same age upon whole casein. Rats upon the arginine-histidine-free amino acid mixture were neither able to grow nor to maintain body weight, but promptly and continually lost weight. The addition of histidine to such a ration resulted invariably in an immediate resumption of growth, at a rate almost or quite equal to that of animals upon normal diets.

Experiments designed to determine the minimum histidine requirement showed that the addition of 0.1 gm. of histidine monochloride per 100 gm. of food (corresponding to approximately 0.5 per cent of the protein when the monochloride is expressed as free histidine) usually sufficed for *maintenance*. 0.2 to 0.3 gm. of histidine monochloride per 100 gm. of food permitted *moderate growth*, while 0.5 gm. (equivalent to 2.5 per cent of the protein, the proportion of histidine normally present in casein) occasioned *a practically normal rate of increase in body weight*. The addition of histidine to the ration was followed in every case by a pronounced increase in food consumption. This occurred whether the amino acid was mixed with the diet, or was fed separately.

In contrast to the behavior of histidine, the addition of arginine to the deficient diet exerted no perceptible influence upon growth, even when the quantity added was more than equivalent to the sum of the arginine and histidine present in native casein. The animals continued to lose weight as rapidly as before the addition of the amino acid. Nor could growth be induced by arginine in animals upon the minimum maintenance allowance of 0.1 per cent of histidine monochloride. We are therefore forced to the conclusion that, contrary to the observations of Ackroyd and Hop-

kins,¹² *arginine and histidine are not mutually interchangeable in metabolism.* The experiments have no bearing upon the question of the indispensability of arginine, since it is quite likely that the Kossel-Kutscher method of precipitation does not remove arginine as completely as it does histidine.

Attempts to satisfy the histidine requirement by the inclusion in the diet of creatine or creatinine gave negative results. Despite the fact that creatinine contains the glycoyamidine ring, closely related to the imidazoles in structure, and that creatine is readily transformed into creatinine, neither substance was able to replace histidine.

The investigation is being continued and extended to other imidazole derivatives. We are also determining the influence of the diamino acids upon the tissue concentration and urinary excretion of purines, allantoin, creatine, and creatinine.

AMINO ACIDS IN NUTRITION.

VIII. THE INDISPENSABILITY OF PROLINE FOR GROWTH.

By BARNETT SURE.

(From the Department of Agricultural Chemistry, Agricultural Experiment Station, University of Arkansas, Fayetteville.)

After more than 50 experiments involving some 200 animals for the study of the rôle of proline in nutrition had been employed, two experiments were finally struck which clearly show the limited synthetic capacity of the animal organism towards the pyrrolidine nucleus of the protein molecule; namely, proline. Since edestin was found to be deficient in cystine and lysine, and since gelatin, although it is deficient in cystine, tyrosine, and tryptophane, was found to supplement edestin very remarkably in the presence of cystine,¹³ experiments were initiated for the purpose of finding out the possible supplementary nature of gelatin, employing edestin as the protein for this investigation and keeping in mind that gelatin is abundant in proline and arginine. Four sets of experiments were started, employing edestin at 9 and 6 per cent levels of

¹² Ackroyd, H., and Hopkins, F. G., *Biochem. J.*, 1916, x, 551.

¹³ Sure, B., *Am. J. Physiol.*, 1922, lxi, 1.

protein intake; the control experiments containing 0.4 per cent cystine and 0.4 per cent lysine, and the other rations 0.4 per cent proline in addition. All the rations were fortified with an abundance of all the other dietary factors, so that proline was the only variable constituent. On the 9 per cent edestin rations no particular advantage was derived by the addition of proline, but the response to proline was very striking on the 6 per cent edestin levels. The experiments were conducted with young albino rats in groups of four, beginning at weaning time, and were carried on for 20 weeks. Accurate food consumption records were kept throughout the period of experimentation. Two animals on the 6 per cent edestin-cystine-lysine ration made very poor growth, while two animals on the 6 per cent edestine-cystine-lysine-proline diet made practically normal growth. For 3 months it was not apparent whether two animals on the proline ration were doing much better than the control experiment. At that point arginine to the extent of 0.4 per cent of the total ration (as a common amino acid) was added to the rations of these groups of animals, which were confined in individual cages, and quantitative records kept of their individual food consumption. Calculating the gain in weight per gram of protein intake, it became very evident that the response to proline in the presence of arginine was very remarkable. Responses to proline were then secured on 6 per cent edestin rations in the presence of cystine and lysine in some animals, and in the presence of cystine, lysine, and arginine in others.

Proline is considered by the author as an essential amino acid for growth.

AMINO ACIDS IN NUTRITION.

IX. THE RÔLE OF ALANINE AND INDOLE IN THE SYNTHESIS OF TRYPTOPHANE BY THE ANIMAL ORGANISM.

By BARNETT SURE.

(From the Department of Agricultural Chemistry, Agricultural Experiment Station, University of Arkansas, Fayetteville.)

Since tryptophane was found to be the determining growth-limiting factor in the proteins of corn, an attempt was made to find whether tryptophane, which is indole-alanine, could be syn-

thesized by the animal organism when indole and alanine (introduced in the form of the leucine fraction from a hydrolysis of zein) are incorporated in the diet. The results of the experiments show that the albino rat is unable to perform such a synthesis when the two components of the tryptophane molecule are administered orally.

ON THE ABSORPTION OF ORGANIC COLLOIDS BY THE INTESTINAL MUCOSA.

By A. B. MACALLUM.

(From the Department of Biochemistry, McGill University, Montreal, Canada.)

Except in the case of fats and soaps, little has been determined regarding the mode of absorption of organic colloids by the epithelial cells of the intestinal mucosa and the manner of transfer through the cells to the interior of the villi. This is due to the fact that there are no microchemical stains which will localize the absorbed proteins as the fats and soaps are revealed by dyes (Sudan III and scarlet red) and osmic acid in the cells.

Some comprehension of the forces involved in this absorption may be obtained from a study of the results of feeding guinea pigs and rabbits, the former more especially, with fresh undiluted egg yolk for a day or more, after they have been kept without food for 24 to 48 hours. The yolk is, in a fluid condition, introduced into the esophagus by a pipette and as much as 10 cc. may thus be given three times a day. Thus given, and in such amounts, the yolk, practically unaffected by gastric digestion, reaches the intestine and immediately comes in contact with the tips of the villi, the cells of which begin at once to take it up and transfer it to the underlying tissues, but as the latter do not absorb it as fast as the cells deliver it to them an accumulation of it occurs at their bases which at the end of 24 hours is so great that the epithelial layer at the tips is "ballooned" or raised to a height several times the long diameter of the normal cells above the "basement membrane," and the cells are flattened by the pressure to which they are subjected internally. They continue to absorb it and at the end of 48 hours or more the "balloons" at the tips of all the villi are ruptured and the contents escape into the intestinal cavity, but before this

rupture occurs some of the absorbed material is transferred through the adenoid tissue to the lacteals which are in consequence greatly distended and their contents contain some at least of the proteins as well as the fats of the absorbed yolk.

A careful examination of the epithelial cells, both in the fresh and in the fixed condition, during the early stages of this absorption makes it evident that in this absorption the cells are active, not passive elements. The contents of the yolk spherules, which are all disintegrated, come into intimate contact with the free borders of the cells, the proteins dissolve in the protoplasmic processes which also take up the fat particles which are almost of ultramicroscopic size and both proteins and fats are transferred through the pores of the basement membrane to the interiors of each cell. Here the cytoplasm dissolves the proteins or enters into intimate relations with them and they diffuse towards the base of the cell where they are set free in a concentrated form with such amounts of the lipoids as are transferred there in a micellar condition. In this passage through the cell the proteins may lag behind the lipoids and as a result one may find in certain of the cells, particularly in those at the sides of the tips of the villi, spherules of protein, of 3 to 4 μ in diameter, free from lipoids, lying in cavities of the cytoplasm which is, however, setting free proteins and lipoids at the basal end of the cell.

The "ballooning" of the epithelial covering of the villi and the great distention of the lacteals indicate that in the transfer of the proteins and the lipoids to the lacteals, the epithelial cells and the cells of the underlying adenoid tissue, work against a pressure which cannot be classified as osmotic pressure as generally understood. Probably the force known as intrinsic pressure plays a very important part in this transfer.

The interpretation of these results has a bearing on the transfer of proteins and lipoids from the blood to the tissues and on the diffusion of these through and from the cells of the tissues.

DIGESTIBILITY OF PROTEINS AND THE ISOELECTRIC POINT.

By A. B. HERTZMAN AND H. C. BRADLEY.

(From the Department of Physiological Chemistry, University of Wisconsin, Madison.)

Foreign proteins, such as albumins and globulins, digest in autolyzing liver brei at characteristic pH values. These values correspond closely to the isoelectric points of these proteins. This indicates that the native protein must be converted into its acid salt or the ion of such a salt before it is substratum. Thus edestin is found to digest at pH 7.0; its isoelectric point is 6.9 (Rona and Michaelis). Egg albumin digests at a pH of 5.0; its isoelectric point is 4.8 (Sørensen).

Egg or serum albumins and globulins inhibit autolysis in a pH range where liver itself digests very well. The inhibition is not complete even with large additions of the foreign protein. The inhibition does not affect the final equilibrium attained but does decrease the speed of the reaction and in proportion to the amount of foreign protein added. The effect of the foreign protein is, therefore, to diminish the active mass of catalyst present without altering the mass of substratum in any way. This indicates the formation of a stable protein-enzyme combination which does not proceed to cleavage, but eliminates a fraction of the enzyme from the reaction.

THE MECHANISM OF THE ACTION OF AMINO ACID PROMOTERS UPON ENZYMES.

By E. W. ROCKWOOD.

(From the Department of Chemistry, the State University of Iowa, Iowa City.)

The activity of urease and ptyalin in solution has been measured, alone and in the presence of α -amino acids which act as promoters. The rate of decay of these enzymes has also been measured. The mechanism of the action of the promoters has been studied and found to consist of two factors; one is the prevention of the decay of the enzyme, but the greater part of the action of the promoter is not due to this. There is a specific stimulation of the enzyme by the amino acid. Possible hydrogen ion effects were eliminated by the use of a buffer.

UREASE AND THE JACK BEAN PROTEINS.

BY J. B. SUMNER, V. A. GRAHAM, AND C. Y. NOBACK.

(From the Department of Biochemistry, Cornell University Medical College, Ithaca.)

The jack bean has been shown by one of the authors to contain two crystallizable globulins, a non-crystallizable globulin¹⁴ and a phosphatide, which is a powerful thrombokinase.¹⁵ In addition there is present a pentose gum and a colloidal yellow pigment. Albumin is absent.

It was thought of interest to see if any of the above substances were concerned in any way with the activity of urease.

Methods of extraction, precipitation, and dialysis combined with methods for the differential adsorption of both proteins and urease have led to the preparation of urease solutions free from both carbohydrate and protein.

The properties of the purified enzyme are discussed.

THE ACID PROPERTIES OF HEMOGLOBIN.

BY A. B. HASTINGS, D.D. VAN SLYKE, J. M. NEILL, AND
M. HEIDELBERGER.

(From the Hospital of The Rockefeller Institute for Medical Research, New York.)

At constant pH oxygenated hemoglobin binds more alkali than reduced hemoglobin. Quantitative measurements have been performed on solutions of recrystallized horse hemoglobin in order to determine the magnitude of the effect at varying reactions. The increase in base bound per molecule of oxygen combined has been found to depend on this reaction. A maximum value of 0.7 equivalent of base added per mol of oxygen has been observed at pH 7.6. At lower pH the value diminishes, falling to 0.4 at pH 6.8. The curve expressing the observed variations of the value with pH agrees with that calculated according to L. J. Henderson's hypothesis, that oxygenation and reduction alter the

¹⁴ Sumner, J. B. The globulins of the jack bean, *Canavalia ensiformis*, *J. Biol. Chem.*, 1919, xxxvii, 137.

¹⁵ Sumner, J. B. Sur le cytozome retiré des graines de canavalia ensiformis, *Compt. rend. Soc. biol.*, 1922, lxxxvi, 108.

dissociation constant of one acid hydrogen in the hemoglobin molecule. The observed values agree with those calculated on the assumption that $K_O = 10^{-6.87}$, $K_R = 10^{-8.38}$, where K_O and K_R represent the dissociation constants of the one labile acid group in the oxygenated and reduced hemoglobin, respectively.

The relationships between base-binding power, reaction, and degree of oxygenation of hemoglobin are expressed by the equation

$$[\text{BHb}] = \beta_R [\text{Hb}] (\text{pH} - I_R) + [\text{HbO}_2] \left(\frac{1}{1 + 10^{\text{pK}'_O - \text{pH}}} - \frac{1}{1 + 10^{\text{pK}'_R - \text{pH}}} \right)$$

Base bound by total hemo- globin.	Base bound by hemoglobin in the re- duced state.	Additional base bound as the result of oxygenation.
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$[\text{Hb}]$ = total hemoglobin, $[\text{HbO}_2]$ = oxygenated portion, β_R = molecular buffer value of reduced hemoglobin, I_R = isoelectric point of reduced hemoglobin, $\text{pK}'_O = -\log K'_O$, $\text{pK}'_R = -\log K'_R$.

STUDIES ON OXIDATION-REDUCTION EQUILIBRIA IN SYSTEMS OF ORGANIC COMPOUNDS.

By W. MANSFIELD CLARK, BARNETT COHEN, H. D. GIBBS, AND M. X. SULLIVAN.

(From the Division of Chemistry, Hygienic Laboratory, United States Public Health Service, Washington.)

Electrometric measurements of the oxidation-reduction potentials of bacterial cultures had given highly interesting data but reliability appeared uncertain and, accordingly, a study has been made of indicators which furnish material for a theoretical study of organic systems and a semi independent method of checking electrode data.

A variety of indicators has been studied in a preliminary way and detailed data have been obtained for the sulphonates of indigo and a variety of indophenols. Electrode potentials are a function of the ratio of reductant to oxidant and of the hydrion concentration of the solution. Equations confirmed by experiment express the influence of the several acid or basic dissociation constants. These as well as the so called normal potentials have been determined and reveal in quantitative terms the effects of chemical substitution.

With the data obtained certain zones in the scale of oxidation-reduction potential have been covered by a system of indicators which should be useful in physiological studies.

ALKALOSIS IN DOGS FOLLOWING INJECTION OF HYDRAZINE SULFATE.

By B. M. HENDRIX AND AVA J. McAMIS.

(From the Laboratory of Biological Chemistry, School of Medicine, and the Laboratory of Chemistry, College of Pharmacy, University of Texas, Galveston.)

It has been shown that a decrease in blood glucose is produced in the dog by injection of hydrazine sulfate. This substance is known to produce liver injury.

The work of Sweet and one of us shows that the carbon dioxide-combining power of the blood may be higher than normal in Eck's fistula diabetic dogs. We have supposed that liver injury brought about this alkalosis.

The object of this investigation is: (1). To see if alkalosis follows the liver injury produced by hydrazine sulfate. (2). To see if there is any relation between the change in pH and the alkali reserve, and the appearance of hypoglycemia.

We have found that injections of hydrazine sulfate increase both the pH and the carbon dioxide-combining power of the blood of dogs. This happens not only when one relatively large injection is given, but also when small doses are given at 48 hour intervals. The hypoglycemia develops to a certain extent at least, in either case.

In a general way, the most pronounced hypoglycemia occurs either on the day of greatest alkalosis or on the day following.

SOME VARIATIONS IN THE ACID-BASE BALANCE OF THE BLOOD
IN DISEASE.

BY VICTOR C. MYERS AND LELA E. BOOHER.

*(From the Department of Biochemistry, New York Post-Graduate Medical
School and Hospital, New York.)*

The explanation of the normal and abnormal variations in the acid-base balance of the blood, advanced by Van Slyke,¹⁶ is simple but comprehensive, and has been of very great assistance to us in the study of clinical conditions of acidosis and alkalosis.

As a measure of the acid-base balance we have estimated the CO₂ content and pH of the blood plasma, the former by the method of Van Slyke and the latter by a modification of the Cullen colorimetric technique previously described by us.¹⁷ About 200 specimens of blood have been examined and among these we have encountered conditions falling into each of the nine areas described by Van Slyke. In our experience the pH of normal individuals falls between 7.35 and 7.43, and we are inclined to regard figures below 7.32 and above 7.47 as definitely abnormal. Our series of cases has included eight cases of uncompensated alkalosis and sixteen cases of uncompensated acidosis, the highest pH observed being 7.60 and the lowest 6.98. The eight cases of alkalosis all received sodium bicarbonate therapy, although in three instances the amount given was very small and was probably not the most important factor involved in the alkalosis. Two cases were receiving Sippy treatment. One case had received radium therapy. Vomiting occurred at irregular intervals in five of the cases, and in at least one case was the probable cause of the alkalosis. Several cases also showed a slight rise in temperature. In the eight cases the plasma pH ranged from 7.49 to 7.60 and the CO₂ content from 56 to 83. Alkalosis is not necessarily associated with an abnormally high blood bicarbonate and it seems evident that with continued bicarbonate therapy the CO₂ content may be reduced to a normal value while the pH increases to an abnormal figure.

Of the sixteen cases of uncompensated acidosis, five were diabetic and eleven were suffering from renal insufficiency. The

¹⁶ Van Slyke, D. D., *J. Biol. Chem.*, 1921, *xlvi*, 153.

¹⁷ Myers, V. C., Schmitz, H. W., and Booher, L. E., *J. Biol. Chem.*, 1923, *lvii*, 209.

pH values ranged from 7.32 to 6.98 and the CO₂ content from 3.8 to 57. Two severe nephritics lived for nearly a month in a condition of uncompensated acidosis.

THE FORMS OF COMBINATION OF THE UNSATURATED FATTY ACID IN BLOOD PLASMA.

By W. R. BLOOR.

(From the Department of Biochemistry, Medical School, University of Rochester, Rochester.)

In earlier work it was shown that fatty acids of a relatively high degree of unsaturation were to be found in blood plasma. Since these substances are probably of importance as stages in the intermediary metabolism of the fats and since in blood plasma they are presumably in process of transportation it was desirable to find out the types of compound in which they occurred. The work presented at this time indicates that most of these acids are in combination with cholesterol with a considerably smaller proportion in combination as lecithin (phospholipoid) and still less as fat. The importance of cholesterol in the intermediary metabolism of the fatty acids is thus indicated.

PLASMA PROTEIN AS AN INDEX OF HYDROPLASMA DURING PREGNANCY.

By E. D. PLASS AND L. JEAN BOGERT.

(From the Department of Obstetrics, Henry Ford Hospital, Detroit.)

The protein content of oxalated plasma from normal non-pregnant young women usually ranges from 6.5 to 7.5 per cent (average 7.00 per cent). No noticeable change occurs during the first 2 months of pregnancy, but beginning with the 3rd month there is a gradual diminution of plasma protein which reaches a maximum at the 5th month. During the last few months of pregnancy the dilution tends to be slightly less marked, and there is a further concentration at the time of labor. During the first 2 or 3 days of the puerperium a redilution occurs, followed by a rapid return to normal, which is attained about the end of the 1st week.

In the late toxemias of pregnancy (eclampsia and its allied conditions), the degree of hydroplasmia is generally exaggerated but tends to follow the same curve.

Hematocrit determinations made at the same time as the plasma protein estimations indicate that the relative plasma volume follows inversely the plasma protein concentration, thereby suggesting that the diminution of the latter is to be looked upon as a true dilution phenomenon. Alterations in the concentration of any serum or plasma constituent during pregnancy may therefore be attributable to variations in blood dilution.

VARIATIONS IN THE BLOOD CHOLESTEROL AND PHOSPHATIDES OF RABBITS DUE TO PREGNANCY.

By EMIL J. BAUMANN AND O. M. HOLLY.

(From the Laboratory Division, Montefiore Hospital, New York.)

The increased cholesterol content of human blood during the latter part of pregnancy has been confirmed from so many sources that it may be taken as an established fact, but its significance is still not well understood. Recently it has been observed that the blood phosphatides are also increased in pregnant women.

In pregnant rabbits, the quantities of these substances in the blood show quite different variations from those occurring in human pregnancies. No rise in either the blood phosphatide or cholesterol values is observed; on the contrary, a marked decrease in both occurs, averaging about 40 per cent in the seven animals studied. The decrease began 15 to 20 days after conception and reached the lowest level 1 to 4 days before parturition, (which usually occurs on the 30th day). The cholesterol and phosphatide values rise again, irrespective of lactation, to the non-pregnant figures, 4 weeks and 1 week after parturition, respectively.

In four pregnant thyroidectomized animals, similar but more marked decreases were observed, averaging 60 per cent of the non-pregnant values. This occurs in spite of the fact that thyroidectomy causes a decided increase especially in the amount of cholesterol and to a lesser extent, of the phosphatides of the blood. The return to the non-pregnant values occurs in a shorter time in thyroidectomized animals than in normal animals—usually within 1 week.

In a few animals we have found that the cholesterol and phosphatides of fetal blood are perhaps slightly higher (15 per cent) than in maternal blood; certainly not lower, as has been found in man.

The decreased cholesterol and phosphatide contents of the blood may be regarded as due to a withdrawal of these substances from the mother to the fetus, to meet the requirements for the very rapid growth of the embryos that occurs in the last 2 weeks of pregnancy.

FURTHER OBSERVATIONS ON CHEMICAL CONSTITUENTS OF SALIVA.

BY J. LUCIEN MORRIS AND CHARLES T. WAY.

(From the Department of Biochemistry, Western Reserve University School of Medicine, Cleveland.)

Supplementing earlier studies of normal salivary secretion, observations were made on pathological cases. Subjects were chosen which exhibited abnormally high concentration in the blood of substances analyzed in saliva. Special attention was paid to urea and uric acid in nitrogen retention cases, such as chronic nephritis, mercuric chloride poisoning, lysol poisoning, and to glucose in diabetes. The amount of blood urea ranged up to more than ten times normal, glucose as high as five times normal, and uric acid to a maximum of somewhat over twice normal.

Glucose results can be briefly summarized: No matter how high the blood sugar concentration in the cases observed, the amount of reducing substance present in saliva was never more than a trace, nor was that trace more than was present in the saliva of subjects having normal blood sugar.

Salivary urea tended to increase with blood urea. However, in terminal nephritis the amount of urea eliminated through the salivary glands did not continue to increase and in some cases was actually less than normal. This was apparently due to failure of the glands to function, possibly because of general failure of body function. In anuria following lysol poisoning, salivary urea percentage exceeded the blood percentage.

Pathological cases emphasized the difference between uric acid and urea secreted in response to various stimuli. In a few

cases, salivary uric acid was definitely concentrated beyond the urea content. In most pathological cases studied the uric acid was diminished markedly, often even to the point of disappearance. Administration of pilocarpine was followed in every case by more marked increase in salivary uric acid than in urea. Such stimuli as chewing caused negligible increases in uric acid, even though volume and urea were usually much increased. This observation confirmed the earlier conclusion that uric acid, more than any other constituent, represents metabolic cellular activity as contrasted with the filtration mechanism.

CHEMICAL CHANGES PRODUCED IN THE BLOOD BY FASTING AND SUBSEQUENT REFEEDING.

BY SERGIUS MORGULIS AND A. C. EDWARDS.

(From the Department of Biochemistry, College of Medicine, University of Nebraska, Omaha.)

The non-protein and urea nitrogen of the blood of fasting dogs usually increases during the early stages of the inanition and then remains at a more or less fixed level until the extreme stage is reached, when a new and much greater increase in nitrogen occurs. The amino acid nitrogen either remains constant all through the duration of the fast or it may diminish slightly at first to rise once more during the latter part of the fast. The blood uric acid increases progressively during inanition. The creatinine remains constant, but the creatine, following a diminution which may occur at an early stage of the fast, rises rapidly in the ultimate stage. The blood sugar and chlorides which usually diminish in the course of the first half of the fasting period increase again during the later stages of inanition. At the time the body weight of the dogs has lost about 40 per cent or more the blood sugar level may even exceed the normal. With but one exception, the undetermined nitrogen and the percentage of total solids in the blood of our dogs increased during inanition.

On repeating a fast, the blood changes become less pronounced or even fail altogether to appear. The total solids, however, increase as they also did during the first fast.

Fasting without water produces the same effects as fasting with a free supply of drinking water, but the changes occur more quickly and are more pronounced when both food and water are being withheld.

Upon feeding the dogs after a protracted fast extensive changes occur in the blood. The non-protein and urea nitrogen, the uric acid, and creatine decrease rapidly in the first few days of realimentation. The decrease is particularly striking in the non-protein and in the urea nitrogen moieties. As the refed animals, however, have gained 35 to 45 per cent in weight the urea and total non-protein nitrogen begin to increase again tending to return to the original level when the prefasting body weight is about restored. The amino acid nitrogen undergoes slight changes, while the creatinine content of the blood is entirely unaffected by the refeeding. During realimentation the total solids of the blood also fall abruptly. In general the composition of the blood approaches the normal condition as the original body weight of the previously fasted dogs is restored.

THE GLUCOSE EQUIVALENT OF INSULIN ON DEPANCREATIZED DOGS.

By F. N. ALLAN.

(From the Physiological Laboratory, University of Toronto, Toronto, Canada.)

Completely depancreatized dogs were kept on a daily ration of 500 or 600 gm. of meat and 100 gm. of cane-sugar. They were also injected daily with insulin. The glucose balance was compared for periods in which varying quantities of insulin were given and the glucose equivalent of the latter calculated by dividing the change in the glucose balance by the change in the number of units of insulin administered. It was found to vary with the doses of insulin, becoming progressively smaller as this was increased. By plotting glucose equivalents against units of insulin a curve was obtained which may be used for determining the glucose equivalent of an unknown sample of insulin. The falling off in the glucose equivalent with increase in unitage is not mainly due to excretion of insulin at higher dosages since injection of constant amounts of insulin daily with varying amounts of carbohydrate give equivalents which increase as the carbohydrate is increased.

INSULIN FROM FISH.

BY N. A. McCORMICK AND E. C. NOBLE.

(From the Physiological Laboratory, University of Toronto, Toronto, Canada.)

Continuing the work on the source of insulin from fish, it has been found that easily removable principal islets exist in many other common bony fishes besides *Lophius* and *Myoxocephalus* and that in the cod (*Gadus callarius*) and the halibut (*Hippoglossus hippoglossus*) large islets occur in close relationship to the gall bladder. These have been removed in large quantities of fish and extracts made by a process which consisted in repeatedly extracting the pulpified islets with about 70 per cent alcohol, containing about 0.3 mineral acid. Alcohol was removed from the extract either by evaporation *in vacuo* or by a current of air, the fat removed by means of ether and the fat-free solution then placed in a boiling water bath. The resulting solution can be used with perfect safety for clinical purposes, but it is desirable to purify the insulin further by applying Dudley's picric acid process and then decomposing the picrate with hydrochloric acid. By these methods large yields of insulin have been obtained at relatively small cost from large quantities of fish. Expressing the yield in terms of clinical units (a clinical unit being one-third the amount of insulin required to lower the blood sugar of a normal fasted rabbit to convulsive level in 4 hours) the following number of units per gm. of principal islets have been obtained on different occasions—cod 22.7, 11.3, 12.7, 13.6, 20.2, 14.7, 19.3, and 17.0. The variability in these yields is due to deliberate alteration of the temperature and method of preservation of the islets before delivery at the laboratory. In one case the material was kept for 17 days at room temperature before being worked up and the yield was only 5.8 units. The yield from the principal islets of pollack in one case was 35 units per gm. of islets; from halibut 18 units; from hake 15 units; from haddock 28.3 units.

It was also observed in several of these fish that extracts of liver prepared by the above method had a marked hyperglycemic effect. Extracts of zymogenous tissue had practically no effect, but if any, they produced a slight lowering (possibly because of the islets in them).

THE EFFECT OF INSULIN ON THE OXYGEN SATURATION OF HEMOGLOBIN.

By J. M. D. OLMSTED AND A. C. TAYLOR.

(From the Department of Physiology, University of Toronto, Toronto, Canada.)

Having noticed that arterial blood in rabbits and cats during the early stages of an insulin convulsion is venous in character, the percentage saturation of hemoglobin with oxygen was determined in decerebrated cats, at half hour intervals after injection of insulin. At the same time variations in the respiratory exchange were recorded. The hemoglobin saturation remained constant for 3 hours until an hour before the appearance of convulsions when it fell from 88 to 80 per cent. At approximately the same time the ventilation of the lungs and the volume of expired CO₂ were both slightly increased, while the volume of O₂ consumed was diminished, causing the R.Q. to rise abruptly to a value slightly above 1. At the beginning of convulsions the oxyhemoglobin had fallen to 75 per cent; ventilation, expired CO₂, and O₂ consumption all fell to a very low value, and the R.Q. became 0.91. Following the convulsions the oxyhemoglobin returned to nearly its normal value, ventilation, expired CO₂, and O₂ consumption were all increased far beyond the normal, while the R.Q. fell slightly to 0.85.

INSULIN IN TISSUES OTHER THAN PANCREAS.

By C. H. BEST, R. G. SMITH, AND D. A. SCOTT.

(From the Insulin Division, Connaught Laboratories, University of Toronto, Toronto, Canada.)

Very soon after the isolation of insulin from the degenerated pancreas of the dog, the discoverers attempted to prepare it from other tissues of this animal. The results obtained with liver and spleen were negative, while those with thyroid or thymus were inconclusive. The demonstration of insulin in blood has been reported by Best, Scott, and Banting, and two of us have reported its presence in various other tissues.¹⁸ We have obtained results

¹⁸ Best, C. H., and Scott, D. A., *J. Am. Med. Assn.*, 1923, lxxxi, 382.

which furnish additional proof that the active material present in tissues other than the pancreas is insulin. The active material produces a marked lowering in the blood sugar of normal rabbits and diabetic dogs. It enables glycogen to be deposited (approximately 6 per cent) in the livers of completely depancreatized dogs. The general condition of these dogs is greatly improved by the therapeutic administration of insulin containing extracts of blood or thymus gland. Extracts of tissue other than pancreas have on numerous occasions produced typical insulin convulsions in normal rabbits. The convulsions are alleviated by dextrose.

Insulin is present in reduced amounts in the tissues of completely diabetic dogs. The extracts of diabetic tissues produced the typical insulin effects on normal rabbits and on diabetic dogs. The amount of insulin in diabetic tissues by the method we have used is more than half that obtained by the same method from normal tissues. Obviously, the insulin must exist in diabetic tissues in a comparatively unavailable form.

We have analyzed various tissues from six normal and six diabetic dogs. Our results indicate that all the muscle tissue of the animal contains at least twenty times as much insulin as the total weight of pancreatic tissue. The blood of a dog appears to contain at least five times as much insulin as the pancreas. In some experiments the liver tissue of an animal yielded nearly as much insulin as was extracted from the pancreas by the same method. The active blood sugar reducing principle was extracted from brain, submaxillary, thyroid, and thymus glands, heart muscle, blood, lung, liver, spleen, kidney, bone, and skeletal muscle and, as previously reported, from the urine.

We have been able to keep a diabetic dog, in which less than 1/800 (0.018 gm.) part of its pancreas was found at autopsy, alive for 8 weeks. No insulin was administered and no dietary precautions observed. Our calculations show that the blood of the animal contained approximately 15 units of insulin at the end of this 8 week period.

The tissues of animals which have been subjected to ether anesthesia appear to contain very much less insulin than normal or diabetic tissues. This is interesting in view of the well known effect of ether upon carbohydrate metabolism.

ON THE PURIFICATION OF INSULIN.

BY H. A. PIPER, R. S. ALLEN, AND JOHN R. MURLIN.

*(From the Physiological Laboratory of the University of Rochester,
Rochester.)*

From extracts of pancreas prepared by perfusion and percolation we have been able invariably to secure a potent preparation of insulin associated with a grayish white precipitate which gives none of the ordinary reactions for protein (biuret, Millon's, xanthoproteic, Hopkins-Cole, etc.). From extracts, also, prepared according to a technique which will be described, we have obtained the active material in this form but not invariably. The determining factors seem to be: (1) the amount of extraneous proteins extracted; (2) the exact pH at which these proteins are precipitated; (3) the reaction at which the insulin together with a residuc of proteins is precipitated with sodium chloride; and (4) the reaction at which concentration and final precipitation are effected. The precipitate, when it contains the active agent is quite insoluble in distilled water, and in acid up to 0.5 N HCl, but is readily soluble in very weak alkali. It readily gives up insulin to the body fluids when injected subcutaneously.

The yield of crude insulin is enormously increased by reextraction of the precipitates. In spite of all precautions, however, much of the insulin is lost in process of purification.

THE RESPIRATORY QUOTIENTS OF NORMAL RABBITS AFTER
THE ADMINISTRATION OF INSULIN.

BY ESTELLE E. HAWLEY AND JOHN R. MURLIN.

*(From the Physiological Laboratory of the University of Rochester,
Rochester.)*

The findings of Dudley, Laidlaw, Trevan, and Boock¹⁹ regarding the effects of insulin upon the output of carbon dioxide and the absorption of oxygen are in accord with effects upon the total respiratory exchange occasionally observed in this laboratory²⁰

¹⁹ Dudley, H. W., Laidlaw, P. P., Trevan, J. W., and Boock, E. M., *J. Physiol.*, 1923, lvii, p. xlvii.

²⁰ Murlin, J. R., Clough, H. D., Gibbs, C. B. F., and Stone, N. C., *Am. J. Physiol.*, 1923, lxiv, 348; Murlin, J. R., Clough, H. D., Gibbs, C. B. F., and Stokes, A. M., *J. Biol. Chem.*, 1923, lvi, 253.

when preparations of crude insulin were given to depancreatized dogs. Dudley's experiments did not permit of a determination of the respiratory quotient, hence they do not warrant the conclusion that more carbohydrate (relatively) is not consumed after insulin. In the experiments referred to with the diabetic dog the R.Q. was invariably higher when the blood sugar was markedly lowered and it was often possible from analysis of the urine to state just how much glucose had been utilized (by combustion or conversion). The interpretation provisionally held, but not published, with regard to the lower total metabolism observed at times was that depressor substances were responsible since only partially purified insulin was used with the animals. Many fatal results upon rabbits have been seen when protein precipitates containing no glucopyretic substance were injected. The object of the present series of experiments was to see whether a purer insulin would produce higher quotients without a depression of the total metabolism and whether a definite impurity which raises blood sugar (*glucagon*) would depress the respiratory quotient. The evidence to date is that both these suppositions are correct. The threshold for effect on the respiratory quotient, however, is not the same as the threshold for effect on blood sugar.

ON THE PREPARATION AND PROPERTIES OF INSULIN.

By M. SOMOGYI, E. A. DOISY, AND P. A. SHAFFER.

(From the Laboratory of Biological Chemistry, Washington University School of Medicine, St. Louis).

Improvements in the method of preparation described a year ago²¹ materially simplify and shorten the procedure, increase the yield and purity of the product, and decrease its cost in time and reagents. The three essential points in our earlier method, and forming the basis of the improved procedure, are: (1) The use of large amounts of strong acid during extraction, which insures solution of the insulin and prevents destruction of proteolytic enzymes; (2) precipitation of the active material by ammonium sulfate; and (3) the precipitation of insulin from semicrude solution by adjusting the reaction to about pH 5.

²¹ Doisy, E. A., Somogyi, M., and Shaffer, P. A., *J. Biol. Chem.*, 1923, *lv*, p. xxxi.

Fresh beef pancreas is finely hashed by passing twice through a motor driven meat grinder. To each kilo of hash are added 20 cc. of 10 N sulfuric acid, and after standing at room temperature from 4 to 12 hours with occasional stirring the extract is filtered through paper (without neutralization). The filtration is rapid. The residue is pressed in a power press, and the press-liquid is filtered. The combined extract is evaporated at low temperature.

When the odor of alcohol is gone, and the volume reduced to about one-tenth (or possibly less) the liquid is allowed to cool to about 20°C. and poured on moistened filter paper. The separated fats are thus completely removed with some protein, while at the reaction of the solution (pH 3 to 4) the insulin remains in the solution.

The clear filtrate is precipitated by half saturation ammonium sulfate, and the active material is separated from the solution of this precipitate by adjusting the reaction to about pH 5.

This first "isoelectric precipitate" contains at least three proteins which may be separated by fractionating at different reactions in alcoholic or aqueous solutions. The active fraction is precipitated best near pH 5, and has been purified to the point that 0.02 or 0.03 mg. of dry material represents a standard Toronto unit. The solubility curves of these protein fractions with changing pH were presented.

FURTHER PURIFICATION OF INSULIN AND ANALYSIS OF THE PRODUCT.

By EDWARD A. DOISY AND CLARENCE J. WEBER.

(From the Department of Biological Chemistry, St. Louis University School of Medicine, St. Louis.)

Fractionation of crude insulin employing alcohol as solvent and the phenomenon of isoelectric precipitation have yielded a product the activity of which lies between 0.015 to 0.025 mg. per Toronto unit (one-third the quantity for a 2 kilo rabbit).

This purified insulin gives a biuret reaction corresponding to an equivalent weight of peptone. By colorimetric procedures, the total nitrogen is 14 per cent. After hydrolysis the ammonia nitrogen is 10 per cent, and the amino nitrogen 70 per cent of the total

nitrogen. The tryosine content is 10 per cent and the cystine 13 per cent. Tryptophane is absent. These figures are only approximate.

Characteristic isoelectric behavior has been observed with solutions containing only 0.05 mg. per cc.

The purified product is rapidly inactivated by trypsin.

SOME EFFECTS OF INSULIN ON THE CARBOHYDRATE AND PHOSPHORUS METABOLISM OF NORMAL INDIVIDUALS.

By N. R. BLATHERWICK, MARION BELL, AND ELSIE HILL.

(From the Chemical Laboratory, Potter Metabolic Clinic, Santa Barbara Cottage Hospital, Santa Barbara.)

1 hour after glucose is taken in tolerance tests (Janney) there is a perceptible drop in the inorganic P of blood plasma. When 40 rabbit units of insulin are given before the glucose there is usually a marked drop in the plasma phosphate and in the excretion of urine P at the time of hypoglycemia. In some cases, the plasma phosphate may first increase and then decrease.

The hourly rates of excretion of P and sugar of two normal individuals living upon a basal diet were determined. During the forenoon the P curve fell and the sugar curve rose, but during the remaining 24 hours they were quite similar, both reaching a peak in the 6 to 8 p.m. period. Insulin in equal doses before breakfast and supper (from 7 to 48 kilo rabbit units in increasing amounts were given) caused an increased excretion of P for 2 and 3 days, respectively. Thereafter the morning rate was markedly decreased. One subject then responded with a decreased rate of excretion for the entire 24 hours. The other subject finally showed a decreased excretion in the rate during the day, but an increased night rate kept the total amount above that for the basal diet. The difference in response of the two subjects is probably referable to the carbohydrate reserves of the body.

The fermentable sugar of the urine decreased by 12 and 28 per cent when insulin was given.

The bearing of these observations upon the formation of the hypothetical hexose diphosphate combination in intermediary carbohydrate metabolism was discussed.

SOME FACTORS INFLUENCING THE RESPONSE OF RABBITS TO INSULIN.

By N. R. BLATHERWICK, M. LOUISA LONG, MARION BELL, L. C. MAXWELL, AND ELSIE HILL.

(From the Chemical Laboratory, Potter Metabolic Clinic, Santa Barbara Cottage Hospital, Santa Barbara.)

Data were presented which showed that rabbits fed the low carbohydrate, base-forming diet of alfalfa hay are less resistant to insulin than are rabbits fed the high carbohydrate, acid-forming diet of alfalfa hay and crushed barley. The greater resistance of animals fed the latter diet appears not to be due to an increase in the glycogen content of the tissues, since animals maintained on both diets and fasted for 24 hours showed practically the same amounts of glycogen in the liver and the muscles.

An "education" to insulin convulsions was also demonstrated; *i.e.*, convulsions are more easily produced after rabbits have had one or more convulsions.

Experiments were cited which indicate that the same amount of insulin per kilo of body weight is effective in producing convulsions even after the rabbits have doubled in weight. These results support the hypothesis that the dosage of insulin varies directly with the body weight and not as the square of the weight as was the belief of some.

In this laboratory, our rabbits are fed a diet of alfalfa hay and are fasted for 24 hours before the insulin test. If no convulsion results from a given dose more insulin is given to produce this result. After convulsions the animals are fed crushed barley in addition to the alfalfa hay; also the following day. The crushed barley is added to replenish the carbohydrate stores of the body. The alfalfa hay must contain a goodly proportion of leaves to be satisfactory. Growing rabbits must gain steadily in weight in order to give reliable results. Our animals are used every 7th day. Rabbits which have never received insulin are given sufficient amounts to produce convulsions before they are used for standardization purposes. This is done to "educate" the animals.

OBSERVATIONS ON INTERMEDIATE CARBOHYDRATE METABOLISM BY MEANS OF INSULIN.

BY CARL VOEGTLIN, EDITH R. DUNN, AND J. W. THOMPSON.

(From the Division of Pharmacology, Hygienic Laboratory, United States Public Health Service, Washington.)

A reliable method for the physiological standardization of insulin has been worked out which is based on the lethal effect produced by the drug in standardized albino rats kept under standard conditions of diet and atmospheric temperature. This method was used to determine the antagonistic effect (against insulin) of certain substances which are generally supposed to be formed in the breakdown of carbohydrates or substances which are believed to be convertible into glucose within the mammalian body. The results obtained indicate that, with certain limitations, the method is quite satisfactory for the study of intermediate carbohydrate metabolism.

THE OCCURRENCE OF A HYPOGLYCEMIA-PRODUCING SUBSTANCE IN BACTERIA.

BY JOHN T. LITTLE, VICTOR E. LEVINE, AND CHARLES H. BEST.

(From the Biochemical Laboratory, School of Medicine, Creighton University, Omaha, and the Connaught Laboratories, Insulin Division, University of Toronto, Toronto, Canada.)

Bacteria are grown in a culture medium free from substances that may contain insulin such as peptone, meat, blood, etc. The simple medium used contains 1 gm. of dipotassium phosphate, 1 gm. of magnesium sulfate, and 2 gm. of asparagine per liter of distilled water. This liquid medium is easily sterilized by boiling. Certain bacteria such as *B. coli* and *B. subtilis* grow very readily in it.

Four flasks each containing 250 cc. of the medium are inoculated and the organisms allowed to grow at 37.5°C. for 4 to 7 days. The medium is then diluted with alcohol so that the content of the latter is 75 per cent, enough sulfuric acid is added to make a concentration of 0.5 per cent. The acid mixture is now allowed to remain at room temperature or at 37.5°C. until upon neutralization, straining, and microscopic examination, the organism has been

found to undergo morphological disintegration. The precipitate which collects at the bottom of the flask is now filtered off and discarded. The hypoglycemia-producing substance is precipitated with 1.25 volumes of ether. After standing 2 or 3 days a precipitate, sometimes of solid consistency and sometimes gelatinous, forms at the bottom. This is removed from the ether by means of a separating funnel and washed three times with 100 cc. of a mixture of equal volumes of 95 per cent alcohol and ether. The washed precipitate, which is very small in quantity is allowed to dry in the air for a few minutes. The precipitate is finally dissolved in 10 cc. of distilled water having a pH of 2.5 and filtered before injection.

The organisms so far tested yield a substance giving a negative biuret and having the power to lower the blood sugar, and to keep the sugar at a low level for a long period. An illustration of the effect on the rabbit of the bacterial hypoglycemic substance (from *B. subtilis*) is appended:

Blood sugars.		
		per cent
Normal.....		0.136
1 hr. after injection.....		0.072
2 hrs. " "		0.054
3 hrs. " "		0.048
4 hrs. " "		0.060

HISTAMINE AS A CONSTITUENT OF SECRETIN PREPARATIONS.

BY ELOISE PARSONS AND FRED C. KOCH.

(From the Hull Laboratories of Physiological Chemistry, University of Chicago, Chicago.)

Comparative chemical and physiological studies on dogs with secretin preparations and pure synthetic histamine led the authors to the following conclusions:

1. Secretin and histamine are similar both chemically and physiologically.
2. The secretin, unlike histamine, is not quantitatively recovered by the phosphotungstic acid precipitation.

3. Secretin, unlike histamine, is not extracted by amyl alcohol in alkaline solution.

4. The depressor and secretagogue actions of secretin and histamine, respectively, are not of the same quantitative order. The former is the better secretagogue and the latter the better depressor.

5. The secretin preparations when properly prepared do not contain histamine in amounts which are of physiological significance.

6. By chemical and physiological assays it was shown that neither secretin nor histamine is absorbed from the duodenal loop and neither is appreciably destroyed by such treatment.

7. The results suggest that secretin preparations contain two activities, a secretagogue and a depressor substance.

THE CHEMICAL REACTIONS INVOLVED IN THE PHYSIOLOGICAL FUNCTIONING OF THYROXIN.

By E. C. KENDALL.

(From the Section of Biochemistry, Mayo Clinic and Mayo Foundation, University of Minnesota, Rochester.)

During the synthetic work leading to the elaboration of thyroxin, a derivative was produced which differed from thyroxin in having one more bond and two less hydrogens. Both of these forms occur in the thyroid gland. The functioning of thyroxin in the tissues is brought about by the molecule oscillating between these two forms. The function of thyroxin is to regulate the capacity of a cell to use oxygen.

The rate of functioning of thyroxin in the tissues is dependent on the physical state of the cell. The alternate oxidation and reduction is regulated by the opening and closing of the pyrrole ring in the tissues. This opening and closing of the ring is dependent on the concentration of positive and negative charges present. It therefore appears probable that thyroxin makes any cell more sensitive to the external, that is, physical conditions.

**A QUANTITATIVE ESTIMATE OF THE CATALYTIC POWER OF
ADRENALIN AND THYROXIN AS CALORIGENIC AGENTS AND
THE RELATIVE RATE OF THEIR DESTRUCTION.**

By WALTER M. BOOTHBY AND IRENE SANDIFORD.

(From the Section of Clinical Metabolism, Mayo Clinic and Mayo Foundation, University of Minnesota, Rochester.)

The administration of both adrenalin and thyroxin produces in the human an excess of heat over and above that which would have been produced had not the substance been given. It is possible to construct plots integrating this increased heat production for given weights of thyroxin and adrenalin. For example, we find in one experiment that 16 mg. of thyroxin injected intravenously in a myxedematous (thyroidless) individual produces above the basal 16,125 calories, or 1,008 calories, per mg. or 589,680 calories per mg. mol. For 0.5 mg. of adrenalin injected subcutaneously there is produced above the basal 25 calories, or 50 calories per mg. or 9,150 calories per mg. mol. Therefore, the relative effectiveness of a molecule of thyroxin as a catalytic agent in the production of heat is 64 times that of an adrenalin molecule.

The various points of the descending limbs of the calorigenic curves of both adrenalin and thyroxin when plotted on semi-logarithmic paper show a remarkable tendency to lie upon a straight line. In a large proportion of the experiments the points are within ± 5 per cent of such a line and in a few are directly upon it. This is consistent with an interpretation that the system suffers an essential change of concentration of only one molecular species and that, therefore, the rate of excess heat production is dependent directly upon the concentration of thyroxin or adrenalin in the tissues. The duration of time for the decrease in heat production from the maximum to one-tenth of this amount is found by extrapolation to be usually, in the case of adrenalin, between 1 and 4 hours and for thyroxin between 30 and 70 days.

**THE EFFECT OF THYROID AND PARATHYROID DEFICIENCY ON
THE GROSS CHEMICAL COMPOSITION OF THE LONG BONES.**

By FREDERICK S. HAMMETT.

(From The Wistar Institute of Anatomy and Biology, Philadelphia.)

When the thyroid apparatus is removed from albino rats 100 days of age a distortion of the course of differential development in gross chemical composition is exhibited. The nature of the disturbance consists of a combined partial desiccation and a retardation of ossification. The desiccation is shown by the fact of an actual loss of water from the bones. The retardation of ossification is shown by the decrease in percentage of ash. The changes are more marked in the humerus than the femur and in the bones of the females.

When the parathyroid glands are removed there is no alteration in the course of differential development in gross chemical composition in the males. In the females an evident retardation of ossification is produced, but no drift towards desiccation.

THE METABOLISM IN PREGNANCY.**II. CHANGES IN THE BASAL METABOLIC RATE.**

By A. W. ROWE, M. D. ALCOTT, AND E. MORTIMER.

Scattered through the literature, nine cases are recorded, more or less completely studied for the basal metabolic rate during pregnancy; that of Root, with one case, being the latest and most complete. In addition, there are two clinical papers by Baer and Cornell, but the studies are too fragmentary and too much influenced by the psychic factor to require discussion. The present report deals with the careful study of two series of cases.

Series I.—This series was comprised of patients from a large hospital prenatal service, and from private practice.

Series II.—These were cases from an institution for unmarried mothers. The basal metabolism was determined by the use of Benedict respiration units of the closed circuit type. Every precaution offered by control measurements was observed to secure a maximum of accuracy, in both the main measurements and the subsidiary observations. The objective findings may be

tabulated as follows, the recorded figures being averages. The relative basal metabolic rate is determined by comparison of the observed rate with the average of the Harris-Benedict and Du Bois standards.

TABLE I.

	Series I.	Series II.	
No. of cases	25	21	
Age, yrs	28	18	
Weight of child, kilos	3.31	3.20	
Partum.	Ante.	Ante.	Post.
Weeks studied	21	8	5
Blood pressure (systolic)	106	115	114
“ “ (diastolic)	65	76	75
Body temperature, °C	98.0	97.6	97.9
Pulse rate	78	82	67
Respiration rate	17	19	19

TABLE II.

Weekly Rate of Change (Antepartum).

	Series I.	Series II.
Weight, kilos	+0.32	+0.52
Vital capacity, per cent	+0.59	
Basal metabolism (absolute) per cent	+0.90	+1.02
“ “ (relative) “ “	+0.53	+0.60
Difference (absolute—relative)	+0.37	+0.42

SUMMARY.

1. There is an increase in the basal energy output. Further, this is in excess of that conditioned by weight increase.
2. The vital capacity increases.
3. The changes seem to be linear in character and are manifest as early as 30 weeks before delivery.

THE DECOMPOSITION OF GLUCOSE BY BACTERIA.

BY E. GORDON YOUNG.

(From the Biochemical Laboratory, University of Western Ontario, London, Canada.)

Fermentations of pure glucose by a stock laboratory strain of *B. coli communis* yielded the variety and proportion of fermentation products obtained from other strains after gross sowings. By the Chambers micro manipulator single cell isolations have been made and fermentations carried out on these pure line strains. Several types of fermentation resulted, differing in the quantitative aspect of the products formed; lactic acid and carbon dioxide, or acetic and succinic acids predominating. This is taken to suggest that the ordinary bacteriological strain of *B. coli communis* contains cells possessing differing types of metabolism. It is further suggested that glucose may be broken down in fermentation by several different paths of decomposition.

PREPARATION AND PROPERTIES OF AN OVARIAN HORMONE.BY EDWARD A. DOISY, EDGAR ALLEN, J. O. RALLS,
AND C. S. JOHNSTON.

(From the Department of Chemistry, St. Louis University School of Medicine, St. Louis.)

A hormone which causes typical estrus in spayed rats has been prepared from liquor folliculi and whole ovaries. Extraction of the lipoids with hot alcohol (95 per cent) is followed by hydrolysis with sodium hydroxide and evaporation to dryness. The hormone is subsequently extracted from the soaps with chloroform or ether. The chief contaminating substance at this stage is cholesterol. Separation by fractional crystallization has been unsuccessful, but the precipitation of the cholesterol by digitonin does not remove the hormone.

The hormone is not cholesterol and does not give cholesterol color reactions. It is soluble in lipid solvents but insoluble in water. It is stable toward dilute boiling acids and alkali. Dissolved in oil, its activity is not destroyed by autoclaving at 15 pounds pressure for 15 minutes.

Preparations from placenta, liquor folliculi, and ovaries from which the liquor folliculi have been removed, are active; from embryos and corpora lutea, negative.

NUTRITION AND GROWTH ON DIETS HIGHLY DEFICIENT OR ENTIRELY LACKING IN PREFORMED CARBOHYDRATES.

By THOMAS B. OSBORNE AND LAFAYETTE B. MENDEL.

(From the Laboratory of the Connecticut Agricultural Experiment Station and the Sheffield Laboratory of Physiological Chemistry, Yale University, New Haven.)

White rats were given diets in which the amount of available *performed* carbohydrate was so small as to be undetectable. The foods contained various proteins and fats in widely different proportions, along with inorganic salts and sources of vitamins A and B. When the energy was furnished essentially in the form of protein rats grew well to 250 gm. or more. The food intake was not notably large, indicating that "protein calories" are well used. Diuresis and hypertrophy of the kidneys were often noted. Microscopic examination failed to disclose inflammation or degenerative changes. On diets containing different proteins and varying proportions of fats with the other dietary essentials but without preformed carbohydrate, good growth was secured in many instances to large size. The absence of preformed carbohydrate did not prevent or alter the prompt recovery of rats which had declined on foods containing no vitamin A, when this factor was supplied. Rats also grew on diets in which the organic foods consisted essentially of protein and fatty acids. The presence of glycerol did not produce any noteworthy advantage. In some of the experiments with foods extremely rich in fats, without apparent detriment to the animals, the ketogenic-antiketogenic ratio greatly exceeded the limit beyond which ketosis is said to arise in man.

Our experiments indicate that in as far as carbohydrate is required for the intermediary metabolism, particularly for the metabolism of fats and the development of energy in muscular contraction, it can be furnished endogenously throughout the period of growth to adult size.

A complete account will appear in the February issue of the *Journal of Biological Chemistry*.

**GROWTH AND REPRODUCTION UPON SIMPLIFIED FOOD
SUPPLY. IV.**

BY H. C. SHERMAN AND H. L. CAMPBELL.

(From the Department of Chemistry, Columbia University, New York.)

Rats were fed mixtures of ground whole wheat and dried whole milk with sodium chloride and distilled water. Starting with a diet which was already adequate for both growth and reproduction, it was found that a change in the quantitative proportions of the food mixture resulted in improved nutrition as evidenced by more rapid and economical growth, earlier maturity, greater success in reproduction and lactation, and postponement of senility. Criteria for the quantitative measurement and interpretation of such differences in nutrition are considered.

FAT-SOLUBLE VITAMIN IN ADULT NUTRITION.

BY H. C. SHERMAN AND F. L. MACLEOD.

(From the Department of Chemistry, Columbia University, New York.)

Two groups of rats of identical family histories have been kept under conditions, alike in other respects, but with different amounts of fat-soluble vitamin in their food. The smaller allowance of fat-soluble vitamin has sufficed to support normal growth to adult size with apparent good health throughout the youth of the animals, but has failed to support normal reproduction and suckling of the young, whereas in the parallel group receiving a more liberal allowance of fat-soluble vitamin, reproduction and success in lactation were fully normal. The more liberal allowance of fat-soluble vitamin has also resulted in a greatly increased length of life.

STUDIES IN PARENCHYMATOUS NEPHRITIS.

BY S. W. CLAUSEN.

(From the Washington University, School of Medicine, St. Louis.)

Parenchymatous nephritis ("nephrosis") is a widespread systemic disorder characterized by albuminuria and edema, without increase of blood pressure or retention of nitrogenous waste. In a series of twenty such cases, a chronic nasal sinus staphylococcus infection could be shown to be the cause in most instances.

The plasma proteins were invariably low, and the colloidal osmotic pressure correspondingly low. This factor may contribute to the edema.

The surface tension of the plasma (or serum) as determined by the drop-weight method is invariably low in contrast to the remarkable constancy in normal individuals. The degree of lowering accurately reflects the clinical progress of the cases. In no other disease excepting nutritional edema is such marked lowering found.

The surface active material may be prepared from the urine as follows: (1) evaporation at low temperature; (2) dialysis; (3) desiccation of colloidal residue; and (4) extraction of dry colloids with 95 per cent alcohol. The dry extract is a wax, swelling in water, soluble in alkali, and precipitated most completely at a reaction of pH 3.5. It is insoluble in ether or benzene. It is salted out from urine and plasma chiefly in the albumin fraction. Animal charcoal removes it completely from these fluids.

Collodion membranes prepared from 6 per cent pyroxylin are normally impermeable to proteins. When first treated with the waxy material in water, or with urine or plasma from cases of parenchymatous nephritis, the membranes become readily permeable.

A VOLUMETRIC METHOD FOR THE QUANTITATIVE DETERMINATION OF SODIUM IN SMALL AMOUNTS OF SERUM.

By BENJAMIN KRAMER AND I. F. GITTLEMAN.

(From the Department of Pediatrics, the Johns Hopkins University, Baltimore.)

2 cc. of serum are evaporated to dryness on a steam bath and ashed according to the method of Stolte.²² After cooling, the ash is dissolved in exactly 2 cc. of 0.1 N hydrochloric acid and the solution is made alkaline to litmus by adding 10 per cent potassium hydroxide (alcohol-washed), drop by drop. Exactly 10 cc. of potassium pyroantimonate reagent²³ are then added, followed by 3 cc. of 95 per cent alcohol. If precipitation does not begin at once it may be accelerated by stirring with a rubber tipped glass rod. The platinum dish is covered to prevent evaporation. At

²² Stolte, K., *Biochem. Z.*, 1911, xxxv, 104.

²³ Kramer, B., and Tisdall, F. F., *J. Biol. Chem.*, 1921, xlii, 467.

the end of a half hour the contents of the dish are poured into a paraffined 50 cc. centrifuge tube. This is centrifuged for a few minutes and the supernatant fluid is then siphoned off. A 10 cc. aliquot is measured into a beaker. Exactly 5 cc. of concentrated hydrochloric acid are added and the beaker is rotated until the precipitate, which first forms, goes completely into solution. 20 cc. of a 20 per cent solution of potassium iodide are then added. Free iodine is formed and colors the solution a deep brown. The sample is then rapidly titrated with 0.1 N sodium thiosulfate until the brown color begins to disappear. 1 cc. of 1 per cent solution of soluble starch is then added and the titration continued with thorough stirring of the sample between additions until the brown color completely disappears, leaving a clear yellow solution. A similar titration is carried out on 10 cc. of the reagent (potassium pyroantimonate).

The calculation is made as follows:

1 cc. of 0.1 N sodium thiosulfate = 6 mg. of antimony.

1 mg. of antimony = 0.192 mg. of sodium.

(1). The number of cc. of thiosulfate used for 10 cc. of reagent $\times 6$ = mg. of antimony in reagent.

(2). The number of cc. of thiosulfate required for a 10 cc. aliquot of supernatant fluid $\times 6 \times \frac{3}{2}$ = mg. of antimony remaining in the supernatant fluid uncombined with sodium.

The difference between (1) and (2) = mg. of antimony in precipitate.

This multiplied by 0.192 = mg. of sodium in the sample $\times 50$ = mg. of sodium per 100 cc. of serum.

THE CHEMISTRY OF JAFFE'S REACTION FOR CREATININE.

By ISIDOR GREENWALD.

(From the Harriman Research Laboratory, the Roosevelt Hospital, New York.)

After acidifying the mixture of creatinine, picric acid, and sodium hydroxide, both creatinine and picric acid can be quantitatively recovered. Only 1 mol of picric acid appears to be required for each mol of creatinine, although the reaction is not complete unless an excess of picric acid is present. The change from red to yellow, upon acidifying, is not immediate, indicating that the alkali is not an essential part of the colored molecule. The red color seems to depend upon an internal rearrangement within

the molecule of creatinine picrate, possibly with the absorption of water. The reaction is not given by a number of substances chemically more or less similar to picric acid.

THE EFFECT OF MANGANESE ON GROWTH.

By VICTOR E. LEVINE AND HERBERT A. SOHM.

(From the Biochemical Laboratory, School of Medicine, Creighton University, Omaha.)

Plant and animal cells are known to contain small quantities of such elements as copper and manganese, and some lower organisms contain vanadium. These elements, like iron, possess more than one valency and are characterized by marked catalytic properties. Their occurrence in living substance argues for some important function on their part. With this view in mind, experiments with minute quantities of these elements have been undertaken in relation to growth. The experiments with manganese illustrate the method pursued.

The experiments were run in units. In order to secure young rats having the same prenatal nutritional and growth tendencies each unit was made up of all males or females from the same litter. One or two rats served as controls, while the others were given varying concentrations of manganese sulfate, which in terms of manganese represented dilutions of 1:2,000, 1:4,000, or 1:10,000.

The diet used was the McCollum polynuritic diet (vitamin B-free casein 18 per cent, dextrin 71.3 per cent, agar-agar 2 per cent, salt mixture 3.7 per cent, and butter fat 5 per cent). The sodium chloride of the salt mixture carried an iodine content of 0.03 per cent. Orange juice was employed as a source of vitamin B and this was served in the ratio of 10 cc. to 40 cc. of distilled water or water containing manganese sulfate. The initial weight of the rats used varies from 20 to 50 gm. The control animals, but particularly the manganese rats, for the first 3 or 4 weeks suffered from severe diarrhea. During this period the animals lost weight or made very slight gain in weight or none. Occasionally the diarrhea proved severe enough to cause death.

The results of the experiments indicate that the manganese has a marked catalytic effect on growth. The manganese rats proved more active than the controls, their coats of hair were sleeker,

longer, and thicker. These facts are in harmony with the power of manganese salts to accelerate oxidations such as take place with unsaturated oils or in the biologic organism, to increase such biologic activities as autolysis, the formation of antitoxin,²⁴ the growth of plants,²⁵ or the healing of wounds.²⁶

In view of the findings of Evans and Bishop²⁷ in reference to a dietary factor essential for reproduction it was surprising to find that in a few preliminary experiments the manganese rats proved fertile and that the offspring of the manganese rats grew somewhat faster than the average young rat. The only source of the reproductive vitamin in the diet may possibly be the milk products, casein, or butter fat, although according to Evans and Bishop the levels of intake employed for either casein or butter fat would not warrant the assumption of the presence of a factor necessary for normal reproductive function.

THE UTILIZATION OF MARGARIC ACID ESTERS.

By ELMER L. SEVRINGHAUS.

(From the Laboratory of Physiological Chemistry, University of Wisconsin, Madison.)

Two healthy adults were fed 2,500 calorie diets, made up of normal foods low in fat and 100 gm. of glyceryl margarate. Beef suet was used similarly as a control. The fat in the stools was determined by titration of free fatty acid and saponification of neutral fat. The beef fat was utilized to the extent of 97.4 to 97.8 per cent. The glyceryl margarate showed 96.5 to 96.8 per cent absorption. There was a slight increase in the neutral fat of the stools when margarate was fed. "Intarvin" was utilized as well as glyceryl margarate.

The same subjects were fed a diet low in protein and almost free from carbohydrate and fat. After ketonuria had become pronounced glyceryl margarate was added in increasing amounts up to 100 gm. daily. There was no diminution in the ketonuria.

²⁴ Walbum, L. D., and Mörch, J. R., *Ann. Inst. Pasteur*, 1923, xxxvii, 396.

²⁵ McHargue, J. S., *J. Agric. Research*, 1923, xxiv, 781.

²⁶ McDonagh, J. E. R., *Proc. Roy. Soc. Med., Dermatol. Sect.*, 1923, xvi, 66, 70.

²⁷ Evans, H. M., and Bishop, K. S., *J. Am. Med. Assn.*, 1923, lxxxi, 889.

The addition of 50 and 75 gm. of carbohydrate promptly reduced ketonuria. Glyceryl margarate seems to have little or no ketolytic value aside from its glycerol content. It does exert a protein-sparing action in these diets.

A NEW DISTINCTIVE TEST FOR CYSTEINE.

By M. X. SULLIVAN.

(From the Division of Chemistry, Hygienic Laboratory, United States Public Health Service, Washington.)

On the addition of one-tenth volume of normal NaOH to a mixture of 1.2 naphthoquinone-4-sodium sulfonate and amino acids and various thio compounds there is speedily developed a color varying from reddish orange to dark brown, dependent on the concentration of the amino acids, etc. Of the colors thus formed, that given by cysteine is the only one not discharged by sodium hydrosulfite, $\text{Na}_2\text{S}_2\text{O}_4$. Cysteine, unreduced, behaves like other amino acids. On the addition of the hydrosulfite, the brownish red color given by cysteine becomes a finer red. If 5 cc. of a solution of various amino acids, containing approximately 0.07 mg. of N per cc., are treated with 0.3 cc. of a 0.5 per cent solution of the naphthoquinone monosulfonate and then with 5 cc. of a 20 per cent solution of sodium sulfite in 0.25 N NaOH, the cysteine (used as cysteine hydrochloride) alone gives the brilliant red color. The colored compound formed by the union of cysteine and the naphthoquinone monosulfonate is apparently a hydroquinone derivative. Work on the quantitative application of the reaction is in progress.

SURFACE TENSION AND THE DETOXICATION OF FOREIGN ORGANIC SUBSTANCES.

By A. R. ROSE AND C. P. SHERWIN.

(From the Department of Chemistry, Fordham University, New York.)

In the study of detoxication a large number of substances have been synthesized or recovered from the urine and purified. The surface tensions of these are being determined with the twofold purpose: first, to see if the surface tensions and the mode of detoxication can be correlated; and second, to find a rapid means of

selecting the probable structure of two or more possibilities in preparations recovered from urines. The observations of Berczeller on benzoic acid have been confirmed. With some exceptions the detoxicated products have a lesser effect on depressing the surface tension of water than their precursors. This relation is particularly noticeable in the direct readings of saturated solutions, but less so and sometimes obliterated or even reversed when calculated to a millimolar basis.

THE ABSORPTION OF SODIUM BENZOATE AND OF SODIUM HIPPURATE FROM THE SMALL INTESTINE OF THE RABBIT.

By WENDELL H. GRIFFITH.

(From the Laboratories of Physiological Chemistry of the University of Michigan, Ann Arbor, and of the St. Louis University School of Medicine, St. Louis.)

In order to use the quantity of hippuric acid excreted in the 6 hour period after the administration of benzoate as an index of the rate of synthesis of hippuric acid, the rate of absorption of the benzoate from the intestine must be ruled out as a process which might influence the output of hippuric acid in the experimental period. Direct determinations were made of the rate of absorption of sodium benzoate and of sodium hippurate from intestinal loops. In these experiments benzoate was found to be absorbed from two to four times as rapidly as the hippurate. After the oral administration of sodium hippurate in quantities equivalent to 1 gm. of benzoic acid per kilo, 51 per cent was excreted in the 6 hour period. Since this represented the rate of absorption of hippurate from the intestine, and since in the loop experiments benzoate was found to be absorbed from two to four times as rapidly as equivalent amounts of hippurate, it was concluded that the rate of absorption of benzoate was sufficiently rapid to permit a high rate of synthesis of hippuric acid after the oral administration of benzoate with a suitable precursor of glycine.

The rate of excretion of sodium hippurate from the kidneys after its oral administration was found to be markedly increased by the simultaneous oral or subcutaneous administration of glycine. Similar results were obtained after the ingestion of hippurate with gelatin or elastin, proteins which have a high content of glycine.

Little or no effect was observed after the administration of hippurate with alanine or proteins which have a low content of glycine, such as egg albumin, casein, edestin, and glutenin. These experiments with others, made it seem probable that the hippuric acid was largely hydrolyzed to benzoic acid in the intestine. The higher rate of excretion after the administration of hippurate with glycine or with proteins containing glycine was considered to be due to a higher rate of conjugation of the benzoic acid with glycine.

PHOSPHOLIPINS IN YEAST.

By W. C. AUSTIN.

(From the Hull Laboratories of Physiological Chemistry, University of Chicago, and the Department of Physiological Chemistry, Loyola University Medical School, Chicago.)

Starch-free baker's yeast contains 4.5 per cent of total lipin and 0.5 per cent of crude phospholipin. A phospholipin with a N:P ratio of unity was made and found to be a mixture of lecithin and cephalin, with the former in predominance. No sphingomyelin was found. The mixture of lecithin and cephalin was fractionated by forming the cadmium chloride salts and recrystallizing the same. Pure lecithin cadmium chloride was prepared. The cephalin decomposed in the process of purification.

Over twenty-five fractions of phospholipin were obtained and analyzed for nitrogen and phosphorus content, and for the nature of the nitrogen-containing substances produced by hydrolysis. The N:P ratios, while near 1:1, were variable, with nitrogen usually greater than 1 in the ratio. The samples yielded choline, cholamine, and varying quantities of unknown nitrogenous substances on hydrolysis. A more accurate method for the estimation of choline was developed.

THE OXIDATION AND REDUCTION OF BILE PIGMENTS.

By WILLIAM M. BARRY AND VICTOR E. LEVINE.

(From the Biochemical Laboratory, School of Medicine, Creighton University, Omaha.)

Bilirubin absorbs oxygen from the air and is converted into biliverdin. This oxidation requires a definite hydrogen ion concen-

tration. Yellow-brown ox bile, dog bile (obtained by fistula), or human bile (obtained by surgical procedure) becomes green after standing for some time at a pH of 6.4. At a pH above this (6.5 to 8.0) the bilirubin of the bile does not undergo oxidation to biliverdin. The bile as it leaves the liver has a pH about the same as that of blood or a little higher (7.2 to 7.8). The tendency of the gall bladder is to increase the acidity of the bile, for bile in the bladder has a pH ranging from about 5.0 to 7.2. Various local pathological conditions, such as infection, trauma, low blood supply, and excessive dehydration may bring about a local acidosis and thereby cause the appearance in the gall bladder of a dark green bile.

Green bile on exposure to air is changed to yellow bile. The change is due to bacteria. Green bile or solutions of biliverdin do not change in color when rendered sterile by heating. The reduction of biliverdin itself or green bile is also accomplished at a pH of 7.4 to 7.6 by liver or yeast cells. This action is due to the enzyme reductase. The reducing action is accelerated by glucose. Heating yeast or liver cells destroys their reducing power.

Liver bile in view of its hydrogen ion concentration and the reducing tendencies of liver cells is a bilirubin bile. We have never found fistula bile to be dark (stasis) or green (oxidation). In infectious conditions of liver or gall bladder the local acidosis may overcome the powerful reducing effect of the liver cells or of the invading organisms with the resulting formation even in the liver itself of green bile or calculi containing biliverdin. In view of the changes liable to take place in bile on standing, observation on color in medical drainage should be made soon after the bile has been collected.

THE EFFECT OF THE INGESTION OF GRANULAR COMMERCIAL GLUCOSE ON THE REDUCING SUBSTANCE IN BLOOD AND URINE.

BY CHI CHE WANG AND AUGUSTA R. FELSHER.

(From the Nelson Morris Memorial Institute for Medical Research, Michael Reese Hospital, Chicago.)

The work was undertaken to determine whether the analysis of hourly specimens of urine might be substituted for tests on

blood sugar for diabetic suspects. Thirteen normal adults served as subjects in two sets of experiments, using 1 and 2 gm. of granular commercial glucose per kilo of body weight. One diabetic was given the same tests with 0.4 gm. per kilo.

The curves for reducing substance in blood were similar in the two cases, showing a maximum rise of about 50 per cent above the fasting value, with the peak at the 30 minute period, and a subsequent decline. The amount of increase in reducing substance in the urine is insignificant when 1 gm. per kilo is ingested. With 2 gm., however, the average figures are seven times as high. The similarity of the blood values and the divergence of the urine values seem to indicate that 1 gm. per kilo is near the average threshold value, and that any increase of sugar ingested is eliminated through the urine. The diabetic patient, after ingesting 0.4 gm. of glucose per kilo of body weight, showed a percentage increase in blood sugar not greatly different from the normal, although the peak was somewhat delayed, but the urinary excretion was tremendously greater—more than twice as much as the highest normal subject on one-fifth the amount ingested. This significant difference in excretion between diabetic and normal suggests the possibility of substituting the tests on urine for those on blood under well controlled conditions.

**SOME OBSERVATIONS ON SUGAR TOLERANCE, USING GRANULAR
COMMERCIAL GLUCOSE, CHEMICALLY PURE GLUCOSE,
AND CHEMICALLY PURE GLUCOSE PLUS EXTRACT
OF COMMERCIAL GLUCOSE.**

BY CHI CHE WANG AND AUGUSTA R. FELSHER.

(From the Nelson Morris Memorial Institute for Medical Research, Michael Reese Hospital, Chicago.)

Tests were made on the reducing substance in blood and urine of six normal adults after the ingestion of 2 gm. per kilo of body weight of (1) c.p. glucose, (2) granular commercial glucose, (3) c.p. glucose plus alcoholic extract of commercial glucose, (4) extract alone, and (5) control. There was no significant difference in the blood under the first three conditions. The peak occurred in all cases after 30 minutes, and was somewhat higher when extract plus c.p. sugar was used. Extract alone had little effect on the blood sugar.

The values for urine are strikingly different from those for blood. There was little difference between the effect of extract and the control, neither showing significant increase in urinary reducing substance. Both commercial and c.p. glucose showed a peak at the end of 1 hour, but the average increase in the former is nearly three times as great as in the latter. When extract was added to c.p. sugar an even greater quantity was excreted. The average peak was delayed until the end of 2 hours, and was nearly four times as great as that of c.p. glucose alone. Although the amount of sugar contained in the extract is only 1.6 per cent of the total amount ingested, the increased excretion of reducing substance is entirely disproportionate. This suggests that there is some factor in the extract which accelerates the excretion of sugar. It will be interesting to determine the specific factor which exerts this influence. Chemical analyses are now under way, but there are as yet no suggestions to offer.

FURTHER WORK ON DYE-PROTEIN AGGREGATES.

By L. F. SHACKELL.

(From the Physiological Laboratory, University of Utah, Salt Lake City.)

Aggregates of the proteins of egg white with "acid" as well as "basic" dyes have been prepared, which do not lose any dye in passing through a pH range of 2 to 8. The extremes of this pH range are well on the acid and basic sides, respectively, of the isoelectric points of the proteins used. Loeb's contention that acid dyes unite with a protein only on the acid side, and basic dyes unite with the same protein only on the basic side of its isoelectric point, is not supported by the present findings.

According to Bayliss, the free acid of Congo red forms blue adsorption compounds with aluminum hydroxide, thorium hydroxide, leucine, and silk. When any one of these compounds is heated, the color changes to a red. Bayliss believes that this change of color follows upon a chemical reaction with the formation of a salt; and that adsorption is a preliminary to chemical combination. In this connection, the writer has prepared a red Congo fibrin, the minute particles of which turn blue in an acid solution. If, after sedimentation, the supernatant acid be drawn off, and boiling distilled water be poured on the blue particles,

the latter turn red practically instantly. These manipulations can be carried out repeatedly with the same particles without producing any separation of dye. Although it is conceivable that an adsorption compound can be transformed into a true chemical union, it is difficult to see how a chemical compound can, by a single, simple manipulation, be changed back into the original adsorption complex.

In the present work an intimation of the real nature of dyed proteins has been furnished. If a protein is aggregated with a dye, acid or basic, which is readily dialyzable (ponceau, methylene blue), the dye can be virtually completely washed away from the protein with hot water. If, however, the dye does not dialyze (Congo red, night blue), aggregates with proteins can be made which do not yield any dye to boiling water. The conclusion of the writer is that these dyed proteins are not adsorption compounds in the ordinary sense, but that they consist of inclusions of the dye within innumerable minute ultrafilters formed during the coagulation of the protein.

THE APPLICATION OF G. BREIT'S FORMULA FOR CONDUCTANCE THROUGH HETEROGENEOUS MEDIA TO BLOOD.

By J. F. McCLENDON.

(From the Laboratory of Physiological Chemistry, University of Minnesota Medical School, Minneapolis.)

Much work on the electric conductance of blood is confused by the use of alternating currents and failure to take into account the capacity resistance of the cells. The use of direct currents obviates this confusion. The conductance of corpuscles, serum, and various mixtures of serum and corpuscles was measured and G. Breit's formula used to calculate the conductance of the cells.²⁸

Calculations from Ohm's law could not be made accurately. A Wheatstone bridge using calomel electrodes separated from the blood by stop-cocks, filled with agar-gel, gave reproducible results if the current was reversed four times a minute. The resistance of the electrodes was subtracted from the total. Mixtures of corpuscles and serum were made by weighing and dividing by specific gravity. The conductivity vessel was immersed in an oil bath.

²⁸ Breit, G., *Koninklijke Akad. wetensch Amst.*, 1922, xxv, 293.

DISTRIBUTION, DESTRUCTION, AND EXCRETION OF URIC ACID.

By OTTO FOLIN, HILDING BERGLUND, AND C. DERRICK.

ON CERTAIN METHODS EMPLOYED IN THE STANDARDIZATION OF INSULIN.

By GEORGE B. WALDEN AND G. H. A. CLOWES.

CHEMICAL CHANGES IN THE EXPERIMENTAL ATROPHY OF MUSCLE.

By K. K. CHEN AND H. C. BRADLEY.

ANTI-KETOGENESIS: THE IN VITRO KETOLYTIC REACTION.

By P. A. SHAFFER AND T. E. FRIEDMANN.

ON FURTHER OBSERVATIONS REGARDING THE INFLUENCE OF pH RANGE ON FERTILIZATION AND CELL DIVISION IN MARINE EGGS.

By HOMER W. SMITH AND G. H. A. CLOWES.

THE LOCALIZATION OF THE SECRETION OF ACID IN THE KIDNEY.

By A. B. MACALLUM.

A NEW THEORY OF URINARY SECRETION.

By A. B. MACALLUM.

PHYSIOLOGICAL RESPONSE TO EXERCISE.

By ETHEL RONZONI.

ALKATAN.

By J. S. HEPBURN AND R. H. STROH.

